# NOVEL ROLES OF P21 IN APOPTOSIS DURING BETA-CELL STRESS IN DIABETES

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## Dedication

Thank you almighty God for guiding me through this journey! This thesis is dedicated to my son and husband, my sister and mother, my grandmothers, and for all the future Latina scientists (especially at IU School of Medicine).

To Jake and Andy Carretero, you two are my everything and everything that I do is for you. To my sister Raquel and mother Ramona, us women can do anything if we put our mind to it—let's keep believing in our potential and encouraging each other, especially when it feels impossible!

I dedicate this work to my grandmothers, Dolores "Lola" Hernandez and Mary Gonzalez. As first-generation US-born citizens, both women have paved the way for me to have a chance at a higher education and to reach my dreams. These two inspirational women have instilled in me the qualities to persevere through life struggles and educational hurdles. Grandma Lola exemplified deep faith in God; compassion for the less fortunate within the community; endurance to raise thirteen children and four generations; love for the family and drive to keep it united. Grandma Mary is like my second mother and she devoted her life to caring for her grandchildren. Through her impactful time as a K-8 teacher's aid, she was first to teach me the importance of education and the rewards of teaching, mentoring, and inspiring younger generations. I strive everyday to be like my grandmas, and I dedicate all my accomplishments to both of them.

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Overall, it truly took a village to get me where I am today-thank you all!

Angelina M. Hernández-Carretero

Novel Roles of p21 in Apoptosis During Beta Cell Stress in Diabetes

Type 2 diabetes manifests from peripheral insulin resistance and a loss of functional beta cell mass due to decreased beta cell function, survival, and/or proliferation. Beta cell stressors impair each of these factors by activating stress response mechanisms, including endoplasmic reticulum (ER) stress. The glucolipotoxic environment of the diabetic milieu also activates a stress response in beta cells, resulting in death and decreased survival. Whereas the cell cycle machinery (comprised of cyclins, kinases, and inhibitors) regulates proliferation, its involvement during beta cell stress in the development of diabetes is not well understood. Interestingly, in a screen of multiple cell cycle inhibitors, p21 was dramatically upregulated in INS-1-derived 832/13 cells and rodent islets by two independent pharmacologic inducers of beta cell stress - dexamethasone and thapsigargin. In addition, glucolipotoxic stress mimicking the diabetic milieu also induced p21. To further investigate p21's role in the beta cell, p21 was adenovirally overexpressed in 832/13 cells and rat islets. As expected given p21's role as a cell cycle inhibitor, p21 overexpression decreased [$^{3}$H]-thymidine incorporation and blocked the G1/S and G2/M transitions as quantified by flow cytometry. Interestingly, p21 overexpression activated apoptosis, demonstrated by increased annexin- and propidium iodide-double-positive cells and cleaved caspase-3 protein. p21-mediated caspase-3 cleavage was inhibited by either overexpression of the anti-apoptotic mitochondrial protein Bcl-2 or siRNA-mediated suppression of the pro-apoptotic proteins Bax and Bak. Therefore, the

intrinsic apoptotic pathway is central for p21-mediated cell death. Like glucolipotoxicity, p21 overexpression inhibited the insulin cell survival signaling pathway while also impairing glucose-stimulated insulin secretion, an index of beta cell function. Under both conditions, phosphorylation of insulin receptor substrate-1, Akt, and Forkhead box protein-O1 was reduced. p21 overexpression increased Bim and c-Jun N-terminal Kinase, however, siRNA-mediated reduction or inhibition of either protein, respectively, did not alter p21-mediated cell death. Importantly, islets of p21-knockout mice treated with the ER stress inducer thapsigargin displayed a blunted apoptotic response. In summary, our findings indicate that p21 decreases proliferation, activates apoptosis, and impairs beta cell function, thus being a potential target to inhibit for the protection of functional beta cell mass.

Patrick T. Fueger, Ph.D., Chair

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## Abbreviations

| | |
|---|---|
| ANOVA | Analysis of variance |
| Atf6 | Activating transcription factor 6 |
| ATP | 5' adenosine triphosphate |
| BH3 | Bcl-2 homology 3 |
| BSA | Bovine serum albumin |
| cAMP | Cyclic AMP |
| CDC | Centers for Disease Control |
| CHOP | C/EBP homology protein |
| CMV | Cytomegalovirus |
| CREB | cAMP response element binding |
| CT | Cycle threshold |
| DMEM | Dulbecco's Modified Eagle Medium |
| DMSO | Dimethyl sulfoxide |
| ER | Endoplasmic reticulum |
| ERK | Extracellular signal-regulated kinase |
| FFA | Free fatty acid |
| FBS | Fetal bovine serum |
| FLIP | FADD-like IL-1β-converting enzyme inhibitory peptide |
| FOXO1 | Forkhead box protein O1 |
| GFP | Green fluorescent protein |
| GLP-1 | Glucagon-like peptide-1 |
| GLUT | Glucose transporter |

| | |
|---|---|
| GR | Glucocorticoid receptor |
| GSIS | Glucose-stimulated insulin secretion |
| GTP | Guanosine-5'-triphosphate |
| HFD | High fat diet |
| IFNγ | Interferon gamma |
| IL-1β | Interleukin-1 beta |
| IL-6 | Interleukin-6 |
| IRE-1α | Inositol-requiring enzyme-1 alpha |
| IRS | Insulin receptor substrate |
| JNK | c-Jun N-terminal kinase |
| LPS | Lipopolysaccharide |
| MAPK | Mitogen-activated protein kinase |
| NADPH | Nicotinamide adenine dinucleotide phosphate |
| NF-κB | Nuclear factor kappa-light-chain-enhancer of activated B cells |
| PBS | Phosphate buffered saline |
| PDK1 | Phosphoinositide-dependent kinase 1 |
| Pdx1 | Pancreatic and duodenal homeobox 1 |
| PERK | (PKR)-like endoplasmic reticulum kinase |
| PI3K | Phosphatidylinositol 3-kinase |
| PIP2 | Phosphatidylinositol 4,5-bisphosphate |
| PIP3 | Phosphatidylinositol 3,4,5-triphosphate |
| PKB | Protein kinase B |
| PKC | Protein kinase C |

| | |
|---|---|
| PKR | Double-stranded RNA-activated protein kinase |
| PLD | Phospholipase D |
| PM | Plasma membrane |
| qPCR | Quantitative polymerase chain reaction |
| ROS | Reactive oxygen species |
| SEM | Standard error of the mean |
| SERCA | Sarco/endoplasmic reticulum $Ca^{2+}$-ATPase |
| siRNA | Small interfering RNA |
| SOD | Superoxide dismutase |
| T2D | Type 2 diabetes |
| Tg | Thapsigargin |
| TXNIP | Thioredoxin-interacting protein |
| TLR | Toll-like receptor |
| TNF$\alpha$ | Tumor necrosis factor alpha |
| TRX1 | Thioredoxin-1 |
| TUNEL | Terminal deoxynucleotidyl transferase dUTP nick end labeling |
| UPR | Unfolded protein response |
| US | United States |
| Xbp1 | X-box binding protein 1 |

# Chapter I. Introduction

## I.A. Type 2 diabetes and obesity

Approximately 29 million people (or 9% of the population) in the United States (US) have diabetes, and the incidence of this devastating disease has increased drastically in the last couple decades, following a similar trend as the rise in the rate of obesity (Centers for Disease Control and Prevention 2014, 2012, Ogden et al. 2014). Within a 16-year timeframe (1994-2010), the percentage of newly diagnosed patients with diabetes who were also overweight or obese increased from 69.7 to 84.7% (Centers for Disease Control and Prevention 2011). In fact, the Centers for Disease Control and Prevention (CDC) recently proposed that 40% of the US population will develop diabetes in their lifetime. Interestingly, the prevalence of diabetes is greater amongst African American, Hispanic, and Native American populations, with a prediction by the CDC that half of African American women and Hispanic men and women will develop diabetes (Centers for Disease Control and Prevention 2012). This "diabesity" epidemic has also spread across the world in many developing and developed nations.

The estimated national cost of diagnosed diabetes in 2012 was $245 billion, incuding both direct medical costs (72%) and lost productivity (28%) such as unemployment from chronic disability, premature mortality, or reduced productivity at work and home (American Diabetes Association 2013). Type 2 diabetes (T2D), the predominant form of diabetes, contributes to metabolic

syndrome and is known to increase health complications, including cardiovascular disease, renal failure, neuropathy, retinopathy, and limb amputations. In fact, according to the CDC, diabetes remains as one of the leading causes of death in the US, and was noted as the 7$^{th}$ leading cause in 2010 (Centers for Disease Control and Prevention 2014).

Although genetics predispose someone to developing T2D, the major reasons behind the recent rise in the disease are most likely explained by environmental factors, such as excessive caloric intake of food sources with poor nutritional value and more sedentary lifestyle choices that promote excessive fat storage. Currently, it is estimated that about 35% of US adults are obese, and the national map of its prevalence is tightly correlated with the national map for the prevalence of diabetes (Ogden et al. 2014, Ogden et al. 2010, Centers for Disease Control and Prevention 2011). A major outcome of obesity is insulin resistance, and both of these co-factors contribute to an increased risk of pre-diabetes and T2D. About 90-95% of diabetes cases are T2D, and even more disturbing is that today 35% of adults above age 20 in the US are pre-diabetic (with a fasting blood glucose level between 100 to 125 mg/dL) (Cowie et al. 2009, Geiss et al. 2010, James et al. 2011, American Diabetes Association 2012).

Diabetes is a disease of misregulated glucose homeostasis. After a meal in normal, healthy individuals, nutrients and metabolic signals traverse to the pancreas, which is comprised of the endocrine Islets of Langerhans within the exocrine acinar tissue. Specifically, glucose signals the beta-cells within the

islets, via specific glucose-sensing machinery within the cell, to secrete insulin into the circulation to stimulate glucose disposal by peripheral tissues, including muscle, liver, and adipose tissue. In addtion, hormonal factors secreted in response to nutrient ingestion such as the incretin GLP-1 potentiate insulin secretion. However, with insulin resistance, these signals are markedly diminished. In an effort to combat insulin resistance, the pancreatic beta cells compensate by increasing their insulin output, which is dependent on an expansion of beta cell mass and enhanced function. The compensatory responses for the increased demand for insulin production and secretion can activate stress responses that ultimately result in the demise of the pancreatic beta cells, which are pathologically targeted in both major forms of diabetes – type 1 and type 2.

Currently, there are interventions to delay the clinical onset of diabetes. Namely, dietary restrictions in combination with exercise are known to prevent pre-diabetic patients from transitioning to frank T2D, or fasting blood glucose levels equal to or above 126 mg/dL (American Diabetes Association 2012). However, patient compliance of this therapeutic intervention limits the efficacy of this approach. Finding a therapeutic treatment that will completely inhibit the decline of functioning beta cells would prove beneficial to reduce the prevalence and severity of diabetes.

In the following subsections, I will describe the beta cell-centric perspective for the cause of diabetes by discussing the beta cell defects occurring in diabetic individuals, the pathological stressors that target the beta

cell, the stress response mechanisms of the beta cell, and the role of the cell cycle machinery during stress, especially with regards to the cyclin-dependent kinase inhibitor 1a, or p21. Chapters III and IV will detail my thesis research findings regarding a novel role for p21 in activating the instrinsic apoptosis pathway in beta cells, and insights into the molecular mechanisms by which p21 is inducing beta cell death during stress (Hernandez et al. 2013). Finally, Chapter V will highlight p21 as a potential therapeutic target to limit the beta cell defects in diabetes and future studies required to fully elucidate the cellular mechanisms behind p21's deleterious effects on the beta cell.

## I.B. Functional beta cell mass

Functional beta cell mass, which incorporates both beta cell mass and beta cell function, is influenced by a number of processes including insulin secretion capacity, rate of proliferation/expansion of beta cells, other mechanisms that increase beta cell number or size, and rate of cell death (**Figure 1**). The beta cell defects that occur in T2D are due to a decline in both beta cell function and beta cell mass – i.e., a composite fall in functional beta cell mass. In regards to beta cell function, impairments in first phase insulin secretion are evident in patients with overt type 2 diabetes and prediabetes (Kanat et al. 2011, Cheng, Andrikopoulos, and Gunton 2013) . For beta cell mass, autopsy studies of individuals with diabetes and pre-diabetes with impaired fasting glucose provide evidence for a reduction in pancreatic beta cell mass

(Matveyenko and Butler 2008, Yoon et al. 2003, Kloppel et al. 1985, Butler et al. 2003).

Changes in beta cell mass occur through various mechanisms in order to adapt to the cellular and organismal environment. Increased regeneration and replication of existing beta cells are mechanisms that positively impact beta cell mass (Migliorini, Bader, and Lickert 2014, Dor et al. 2004). Neogenesis from neurogenin3+ ductal progenitors, regeneration of intra-islet multipotent pancreatic progenitors, or conversion of acinar cells into beta-like cells are all potential ways to regenerate beta-cells (Migliorini, Bader, and Lickert 2014). However, with aging, the replication and regeneration capacites of beta cells dramatically decline and are at very low levels post-natally (about less than 0.07% of human beta cells undergo BrdU+ proliferation) (Teta et al. 2005). Furthermore, dedifferentiation and apoptosis negatively affect beta cell mass. Recently, dedifferentiation has been brought to light where during beta cell stress, beta cells dedifferentiate into a progenitor state that then is stimulated to differentiate into glucagon-secreting alpha cells of pancreatic islets (Talchai et al. 2012). This mechanism could contribute to the decline in total overall beta cell mass in T2D. Similarly, apoptosis or beta cell death are stimulated by various cellular stressors and also contributes to the decline in mass. In theory, finding methods to inhibit dedifferentiation and apoptosis, while at the same time increase replication, neogenesis, or regeneration of beta cells will preserve or enhance beta cell mass.

Aside from preserving beta cell mass, it is equally essential to maintain or enhance beta cell function to prevent diabetes and diabetes-related secondary complications. Changes in glucose sensing, pulsatile insulin secretion, glucose-stimulated insulin secretion (GSIS), proinsulin processing, and/or expression of genes and proteins required for insulin production, folding, and/or release impact beta cell function. Strong evidence suggests that an impairment in GSIS is evident long before the progression to full-blown diabetes (Ferrannini et al. 2005, Ferrannini et al. 2013). Factors, such as hyperglycemia, that constantly stimulate depletion of insulin granules lead to a loss of early phase insulin secretion, thus, negatively impacting beta cell function.

During the progression to T2D, when peripheral tissues are insulin resistant, beta cell compensation occurs to maintain normoglycemia with the increased demand for insulin. This phase is characterized by hyperinsulinemia as a result of increased beta cell mass and/or functional capacity. Yet, eventually over time, the beta cells can no longer keep pace with the high insulin demand and undergo a switch to decompensation where beta cells begin to undergo cell stress, functional failure, and ultimately death. As the decompensation phase progresses, functional beta cell mass declines resulting in glucose intolerance and hyperglycemia, both characteristics of T2D.

**Figure 1.**

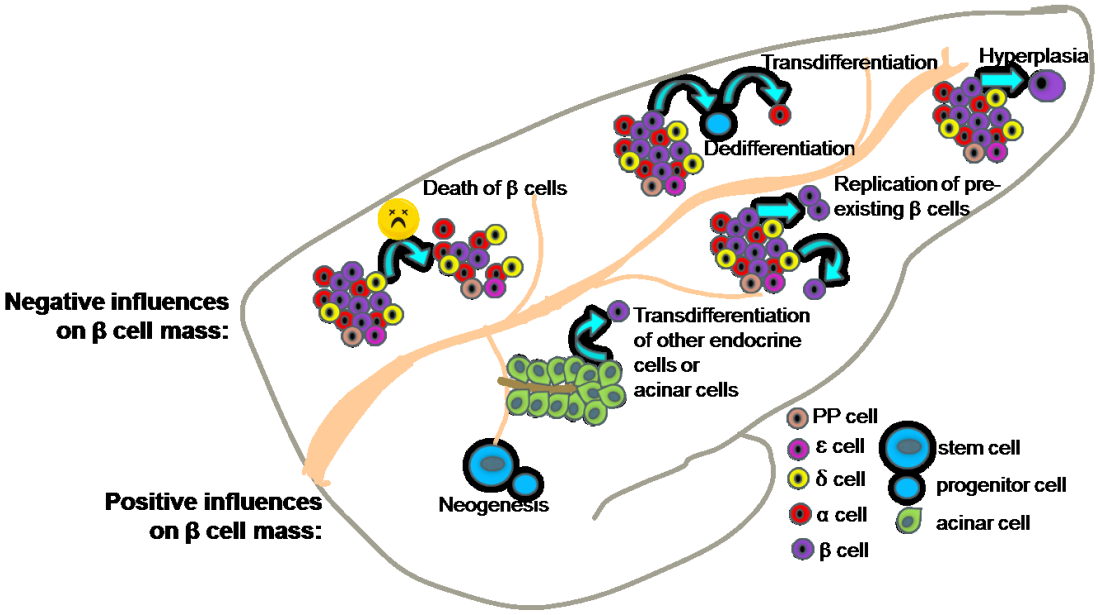


**Figure 1. Overview of the regulation of functional beta cell mass.** Pancreatic beta cell mass is negatively regulated by beta cell death, dedifferentiation, and transdifferentiation. On the other hand, positive influence on beta cell mass include neogenesis, transdifferentiation of other endocrine or exocrine cell types, replication of pre-existing beta cells, and hyperplasia.

## I.C. Beta cell stress in diabetes

The overall research goal of my thesis work is to discover mechanisms that fortify functional beta cell mass. One way to achieve these goals is by studying molecular mechanisms that promote the switch between the compensation and decompensation phases. Some postulated mechanisms of beta cell destruction that promote this decompensation switch include stressors that directly act on the beta cells, such as human islet amyloid peptide, inflammatory cytokines, glucocorticoids, reactive oxygen species, high concentrations of glucose, or glucolipotoxicity, all of which are elevated in obese or diabetic individuals and are thought to contribute to the decline in functional beta cell mass (Kitamura 2013, Halban et al. 2014, Surampudi, John-Kalarickal, and Fonseca 2009). The islet responds to these stressors by activating cellular mechanisms that primarily work to alleviate or repair the negative effects of the stressors (summarized in **Figure 2**). However, ultimately with severe or prolonged activation, these mechanisms are deleterious to beta cell function and mass, resulting in T2D. Although it may be difficult to fully block each of these response pathways, a good therapeutic approach to prevent the loss of functional beta cell mass is to find commonalities in the destructive mechanisms of multiple stressors. Identifying points of convergence in signaling pathways may yield the most effective therapeutic targets for preventing or treating T2D at the level of the beta cell. The following subsections will briefly review critical stressors implicated in the development of T2D and were the focus of my thesis work.

**Figure 2.**

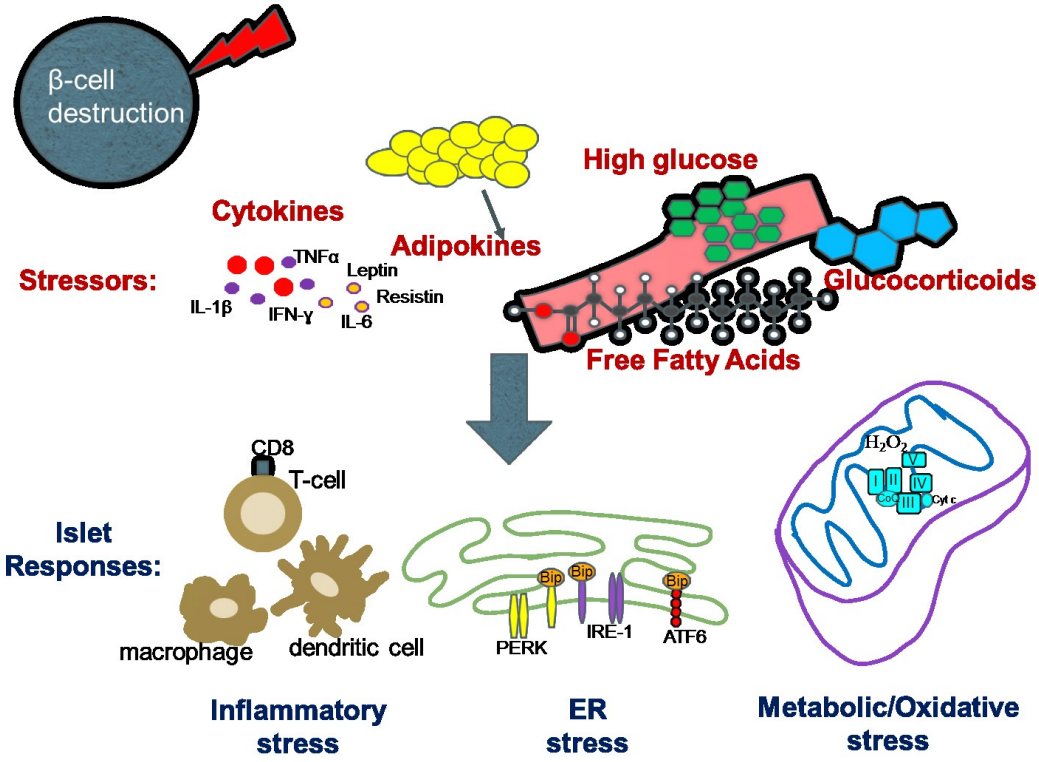


**Figure 2. Summary of stressors in diabetes and the beta cell responses.** Cytokines, adipokines, glucotoxicity, lipotoxicity, and glucocorticoid steroids are all stressors that lead to beta cell destruction. These stressors elicit beta cells to activate inflammatory stress, ER stress, and metabolic/oxidative stress responses in order to combat the deleterious effects. Chronic activation of these responses lead to a decline in functional beta cell mass and overt T2D.

## CYTOKINES

Inflammation is evident in many organs of diabetic patients and underlies part of the pathophysiology within the pancreatic islets as well as other tissues that participate in the long-term complications associated with poorly controlled diabetes. Serum samples of patients with diabetes and/or obesity are indicative of an inflammatory status as there are increased levels of circulating proinflammatory markers (Horvath et al. 2013, Donath and Shoelson 2011, Hatanaka et al. 2006, Spranger et al. 2003, Carstensen et al. 2010, Zhang, Xiao, et al. 2014). Additionally, adipose tissue of obese individuals have increased levels of activated T-cells and proinflammatory adipokines that contribute to the systemic inflammatory status. The inflammatory status of diabetes is also evident within the pancreatic islets as there are elevated levels of cytokine production by

beta cells, along with increased CD68+ macrophage cells or other immune cells residing near beta cells. The beta cell-specific antigen(s) that activates the inflammatory response is still unknown. However, this event entails cytokines that activate the receptors from the Toll-like receptor (TLR) family (TLR2 and 4 in humans) to: 1) auto-stimulate further production of the master cytokine interleukin-1 beta (IL-1β) in an autocrine fashion; 2) recruit further immune cell infiltration by releasing other paracrine-acting chemokines and cytokines; 3) induce fibrosis and apoptosis; and 4) impair insulin production and secretion.

Inflammatory cells recruited to the islet include T helper cells, cytotoxic T cells, macrophages, and dendritic cells. It is yet unknown as to which event occurs first; however, inflammatory cells in the islet cause death of beta cells,

thereby releasing antigens that trigger the recruitment of even more inflammatory cells to “clean up” and engulf islet debris. In either event, at low concentrations of inflammation, IL-1β will activate FLICE-inhibitory protein (FLIP), an inhibitor of the Fas signaling pathway, to induce beta cell proliferation and function in a nuclear factor kappa-light-chain enhancer of activated B cells (NF-κB)-dependent manner that acts through Pancreatic and duodenal homeobox 1 (Pdx1) (Maedler et al. 2006, Schumann et al. 2007). NF-κB is ubiquitously present in cells and upon activation, this transcription factor stimulates expression of genes involved in T-cell development, maturation, and proliferation in order to maintain the proper amount of inflammatory mediators to protect from any antigenic damage. On the contrary, prolonged activation of NF-κB transcriptional activity is deleterious to beta cells as it maintains a consistent abundance of proliferating inflammatory cells and activates inducible nitric oxide synthase expression in beta cells, thereby inducing nitric oxide and beta cell death (as described in more detail below).

In addition to impairing insulin production and secretion in beta cells, cytokines activate death pathways that work through various mechanisms, including but not limited to the activation of apoptosis, the inflammasome and cytotoxicity, all of which impact functional beta cell mass (Padgett et al. 2013, Donath and Shoelson 2011). IL-1β, interferon-gamma (IFN-γ), and tumor necrosis factor-1 alpha (TNFα) are the most prevalent proinflammatory cytokines that are pathologically detrimental to beta cells. Elevated cytokines activate the stress kinase, c-Jun N-terminal kinase (JNK), NF-κB signaling, and Janus Kinase

(JAK)/Signal transducer and activator of transcription (STAT) pathway, while increasing nitric oxide production and reactive oxygen species (ROS) . JNK activates pro-apoptotic transcription factors, such as AP-1, c-Jun, c-Fos, Jun-D, and ATF-2 under proinflammatory cytokine exposure (Eckhoff et al. 2003). Similarily, the JAK/STAT pathway aids in the activation of pro-apoptotic genes (Heim 1999). Nitric oxide and other free radicals induce DNA damage, interfere with mitochondrial iron-sulfur containing enzymes such as aconitase of the tricarboxylic acid cycle, and lead to the increase of mediators from the cyclooxygenase pathways, such as prostaglandins, thromboxane A2, and prostacyclin (Salvemini et al. 1993, McDaniel et al. 1996). Interestingly, repair of nitric oxide-induced DNA damage in beta cells requires JNK, and thus JNK is paradoxically both protective and harmful, depending on the severity or length of activation (Hughes et al. 2009). Although there is some induction of apoptosis through JNK and nitric oxide, cytokines also lead to beta cell death by disrupting ATP homeostasis and inducing cytotoxicity and necrosis rather than by apoptosis (Collier et al. 2006, Steer et al. 2006).

Additionally, increased cytokines are associated with decreasing cell survival responses or insulin signaling. For example, TNFα-stimulated adipocytes or cytokine-stimulated beta cells display decreased phosphorylation of insulin receptor substrate-1 (IRS-1) and IRS-2, which are downstream of insulin receptor activation (Gurevitch et al. 2010, Hotamisligil et al. 1996). Lastly, cytokines such as IL-1β and IL-12 impair GSIS, and when the IL-1 receptor antagonist is used as a treatment in a diabetic rat model, there is improved proinsulin to insulin

processing (Donath et al. 2009, Taylor-Fishwick et al. 2013). As mentioned above, the inhibitory actions of cytokines on insulin secretion are mediated by nitric oxide, as it targets aconitase from the Krebs cycle, thus decreasing ATP production that is necessary for depolarization of the plasma membrane and insulin secretion (Corbett et al. 1991, Welsh et al. 1991). Therefore, increased cytokines result in cell death, inhibition of proinsulin to insulin processing, and impaired GSIS, which overall contribute to the decline in functional beta cell mass.

## GLUCOCORTICOIDS

Obese individuals have elevated levels of cortisol, a glucocorticoid steroid hormone that promotes an anti-inflammatory response upon activation of the glucocorticoid receptor (GR). Additionally, obese and insulin resistant individuals have increased expression of 11-hydroxysteroid dehydrogenase, the enzyme that converts cortisone to the active cortisol (Duplomb et al. 2004, Davani et al. 2000). Further, stress elicits the release of glucocorticoids into the bloodstream to regulate the release of glucose during the fight or flight response. Specifically, glucocorticoids suppress the immune system, stimulate gluconeogenesis and glycogenolysis, and activate genes regulating fat, protein, and carbohydrate metabolism. Upon ligand binding to GR, it transcriptionally represses genes such as NF-κB to promote an anti-inflammatory response. Although glucocorticoids are utilized for their role as an anti-inflammatory agent, caution is rendered as steroids induce insulin resistance and diabetes (Lansang and Hustak 2011,

Ferris and Kahn 2012, Boumpas, Anastassiou, et al. 1991, Boumpas, Paliogianni, et al. 1991, Saksida et al. 2014). Administering corticosterone to C57BL/6J mice via the drinking water for 5 weeks induces glucose intolerance, impairs insulin sensitivity, and causes dyslipidemia (Fransson et al. 2013).

In addition to the deleterious effects that glucocorticoids have on the entire metabolic state *in vivo*, glucocorticoids are also markedly detrimental to beta cells. For example, intracellular cytosolic calcium levels are disrupted in response to glucose when isolated rat beta cells are cultured in increasing doses of corticosterone (Koizumi and Yada 2008). Therefore, the calcium required for first- and second-phase insulin secretion is decreased and does not allow for adequate insulin release from the beta cell. Elevated GR activation via dexamethasone, a synthetic GR agonist, also suppresses GSIS by increasing degradation of glucose transporter type 2 (GLUT2), the primary transmembrane carrier of glucose into beta cells that is necessary to metabolize and sense glucose for stimulation of insulin secretion (Gremlich, Roduit, and Thorens 1997). Additionally, dexamethasone treatment of immortalized rat beta cells and rat islets resulted in activation of FOXO1 transcriptional activity and a FOXO1-dependent inhibition of the expression of Pdx1, a transcription factor essential for the differentiation, function, and maintenance of beta cells (Zhang et al. 2009). Overall, glucocorticoids lead to a decline in beta cell function by disrupting GSIS and the maintenance of beta cells.

Studies in islets using dexamethasone have shown that along with suppressing insulin secretion, chronic stimulation of the GR activates beta cell

apoptosis, primarily through intrinsic mechanisms (Gruver-Yates and Cidlowski 2013, Gruver-Yates, Quinn, and Cidlowski 2014). INS-1 cells (an insulin-secreting rat beta cell line) treated with dexamethasone had increased transferase-mediated dUTP nick-end labeling (TUNEL)-staining, caspase-3 activation, decreased levels of the anti-apoptotic protein Bcl-2, and increased pro-apoptotic Bad and Bim expression (Ranta et al. 2006, Gruver-Yates, Quinn, and Cidlowski 2014). Additionally, stress kinases are upregulated during dexamethasone treatment of beta cells. Both p38 mitogen-activated protein kinase (p38 MAPK) and c-Jun N-terminal kinase (JNK) are activated with dexamethasone treatment, and pharmacologically inhibiting p38 MAPK reduced dexamethasone-induced damage in beta cells (Fransson et al. 2014). Interestingly, treatment with a JNK inhibitor in conjuction with dexamethasone augmented DNA fragmentation and death of beta cells, suggesting that dexamethasone primarily induces damage through the p38 MAPK pathway, and not the JNK pathway.

In addition to regulating stress kinase signaling, glucocorticoids also decrease the redox-regulating ability of beta cells. Dexamethasone upregulates the expression of thioredoxin interacting protein (TXNIP), and decreases the antioxidant thioredoxin protein (TRX1) (Reich et al. 2012). This effect was dependent on p38 MAPK and was reversed by the GR antagonist RU486. Overall, studies utilizing dexamethasone treatment of beta cells *in vitro* indicate that GR activation promotes apoptosis, thus translating into a decline in beta cell mass.

## GLUCO(LIPO)TOXICITY

As mentioned previously, insulin is released into the circulation to stimulate glucose disposal by peripheral tissues. In addition, insulin signals adipose tissue to inhibit lipolysis and stimulate lipogenesis. However, in the insulin resistant stage (pre-diabetes or T2D), these signals are diminished, leading to an accumulation of circulating free fatty acids, and in combination with hyperglycemia, they synergistically act on beta cells to exacerbate death signals (also known as glucolipotoxicity) (Poitout and Robertson 2002, Robertson et al. 2004).

Long-term exposure to saturated free fatty acids (FFA), such as palmitate, is especially harmful to non-adipose tissues (like the beta cells) because it provokes apoptosis (Lupi et al. 2002). FFA can trigger apoptosis by inducing the production of ROS, and also directly disrupting the structure and integrity of the endoplasmic reticulum (ER). Both events then lead to ER stress (described in detail in section I.D.) and the release of ER calcium stores to trigger the intrinsic mitochondrial apoptotic pathway (Kharroubi et al. 2004). Controversy exists as to whether the deleterious effects of a high lipid environment for beta cells are not as robust without the presence of high glucose. This is because the high glucose environment switches the oxidation status of fatty acids by shunting them towards esterification pathways, leading to the intracellular accumulation of FFA.

Similar to lipotoxicity, high concentrations of glucose alone, or glucotoxicity, are also known to induce similar deleterious effects. For example,

glucotoxicity induces oxidative stress by elevating peroxide levels and other ROS in human islets (Robertson 2004). Additionally, glucotoxicity also results in apoptosis of beta cells. One postulated mechanism for this is that high glucose concentrations upregulate the production of IL-1β, which activates inflammatory pathways as mentioned previously and also induces the Fas receptor, a receptor that mediates the extrinsic apoptotic pathway (Maedler, Fontana, et al. 2002, Maedler, Sergeev, et al. 2002). Glucotoxicity-induced apoptosis requires the proapoptotic Bcl-2 homology domain 3 (BH3)-only proteins Bim and Puma, and presenting their absence, beta cells are protected from ROS (Wali et al. 2014). Overall, these mechanisms contribute to the decrease in beta cell mass, however, an emerging consequence of glucotoxicity is that it is a major cause of beta cell dedifferentiation (Weir, Aguayo-Mazzucato, and Bonner-Weir 2013). Aside from decreasing beta cell mass, glucotoxicity also impairs beta cell function and GSIS by inhibiting the expression and activity of protein kinase A, a downstream effector of cAMP signaling that potentiates GSIS (Kong et al. 2014).

Similar to glucotoxicity, *in vivo* models of glucolipotoxicity, including high fat feeding in mice, show increased inflammatory and oxidative stress markers as well as activation of apoptotic pathways. Saturated free fatty acids bind to lipopolysaccharide (LPS) receptors, such as toll-like receptor-4 (TLR4) to activate the inflammatory response and increase IL-1β expression. The exact cellular mechanisms of glucolipotoxicity are still incompletely understood, however, it is evident that there is also activation of stress kinases such as JNK, and ER stress

markers such as phosphorylated eIF2α (p-eIF2α) and C/EBP homology protein (CHOP) (Martinez et al. 2008).

In addition to activating death signals, glucolipotoxicity can interfere with metabolic processes and impair insulin secretion of beta cells (Somesh et al. 2013). Within a week of high fat feeding in C57BL/6J mice (60% kcal from fat) GSIS, is impaired (Stamateris et al. 2013). One fact to note, however, is that at this stage, beta cell compensatory responses are still fully engaged, characterized by increased beta cell proliferation, and hence, increased beta cell mass. Similar to the effects of glucocorticoids, glucolipotoxicity after the compensatory stage decreases glucose uptake into beta cells by reducing GLUT2 expression and also impairs downstream calcium release required for insulin secretion (Somesh et al. 2013, Gremlich et al. 1997). Further, glucolipotoxicity disrupts glucose metabolism by reducing glucokinase and pyruvate carboxylase, two major enzymes that mediate the catabolism of glucose and fatty acid synthesis. As a result, cellular NADPH, GTP, and ATP production are all reduced, thereby disrupting the insulin secretion process. Additionally, there are less intermediates for fatty acid oxidation and an increase in fatty-acyl CoA accumulation to form triglycerides within beta cells – i.e., fatty acids accumulate.

Altogether, glucotoxicity activates inflammatory responses, increases ROS, and interferes with metabolic signaling. Similarly, gluco*lipo*toxicity also activates similar responses, while at the same time induces ER stress, and impairs metabolic processes and beta cell function. Thus, these processes

negatively impact functional beta cell mass and are of great interest for further investigation.

## I.D. Beta cell stress responses

In response to stress, multiple pathways are activated to repair any deleterious effects or are activated to signal the cell to die, depending on the severity of the stress. In this section, I will provide an overview of general death signals and cell stress/survival pathways, and then elaborate on other responses to a specific type of stress, including oxidative and ER stress.

### DEATH SIGNALS

Prolonged or severe activation of cellular stress pathways leads to either apoptosis or necrosis in order to execute the death of the cell. Apoptosis is activated by two distinct, but converging, pathways—the extrinsic and/or intrinsic pathways (**Figure 3**). Regardless of the apoptotic pathway, this form of cell death consists of a series of events that are energy-dependent and that ultimately lead to ordered fragmented DNA and organized apoptotic bodies. The extrinsic pathway is stimulated by extracellular signals that activate the death receptor, releasing cleaved caspase-8 from the DISC compartment that leads to downstream caspase-3 cleavage and ultimately apoptosis, whereas the intrinsic pathway is activated by intracellular stress that increases pro-apoptotic Bax and Bak translocation the mitochondrial membrane. This Bax/Bak localization allows for pore formation and the release of cytochrome c from the mitochondria to form

the apoptosome, which activates the downstream caspase cascade and apoptosis. Anti-apoptotic proteins such as Bcl-2, Bcl-xL, or MCL, however, can also translocate to the mitochondrial membrane to inhibit this process by blocking pore formation/release of cytochrome c.

Necrosis is the other mechanism by which cells undergo death. This form of death is characterized by bursting of the cell and does not activate a specific program. Therefore, it is difficult to detect and block necrosis in cells, and usually it is determined to be necrotic cell death if it does not undergo characteristics of apoptosis. Some stressors that activate alternative death pathways include cytokines and inflammatory stress. Necrosis is characterized by a loss of plasma membrane integrity, random DNA degradation, recruitment of immune cells that elicit inflammation, impairment in the bioenergetics of the cell (e.g., decreased cellular ATP), and initiation of tissue repair mechanisms (Zong and Thompson 2006).

**Figure 3.**

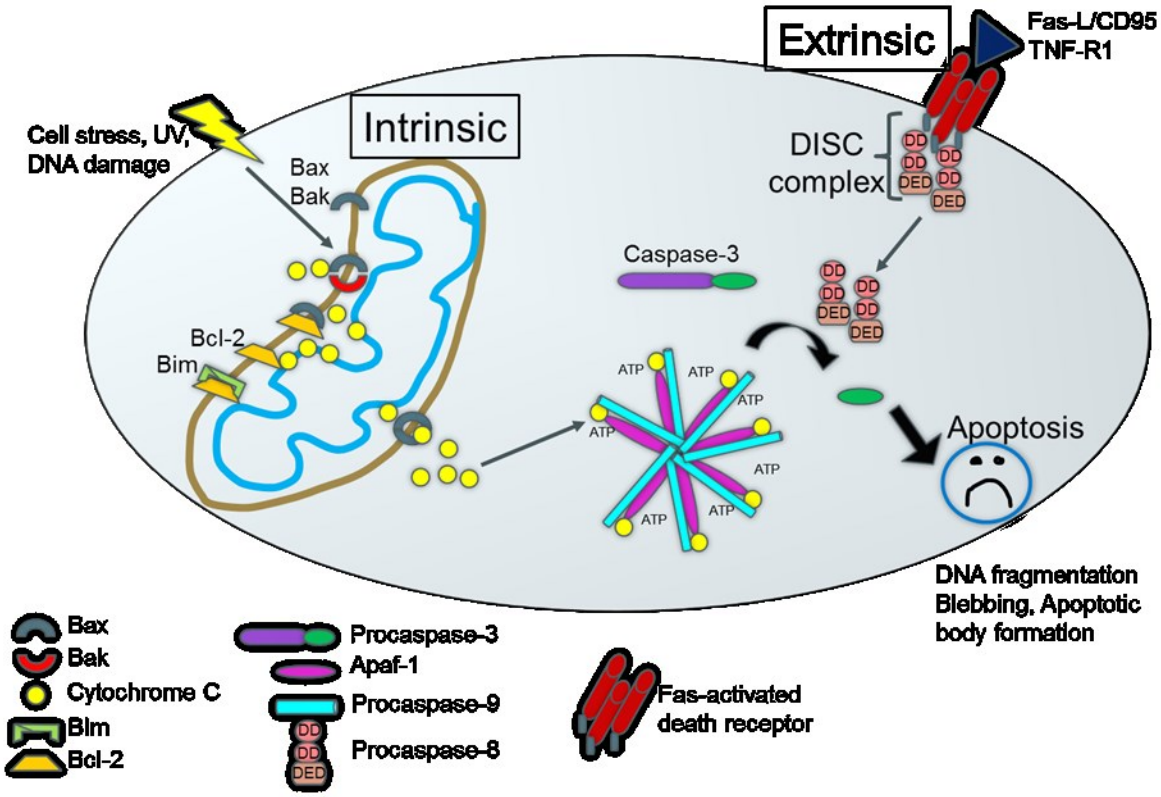


**Figure 3. Extrinsic versus intrinsic apoptotic pathways**. The extrinsic and intrinsic pathways are distinct mechanisms that converge at cleavage of caspase 3 to activate an apoptotic response that involves DNA fragmentation, blebbing, and formation of apoptotic bodies. The extrinsic pathway is stimulated by an external ligand that activates Fas-activated death receptors on the plasma membrane of the cell. Whereas, the intrinsic pathway is stimulated by mechanisms within the cell such as cell stress, ultraviolet (UV) rays, or DNA damage.

## CELL STRESS AND SURVIVAL PATHWAYS

After the initiation of beta cell stress, survival pathways are activated to maintain cellular integrity, function, and viability. However, with severe or prolonged induction of stress pathways, these signals lead to detrimental effects. Interestingly, many stressors stimulate pathways that converge onto a few cellular signaling cascades that involve mitogen-activated protein kinase (MAPK), c-Jun N-terminal kinase (JNK), p38, and Akt.

The MAPK (ERK) pathway regulates proliferation, cell division, and differentiation. In a site of injury, mitogens and growth factors are produced to repair and regenerate the damaged area. Mitogens or growth factors act as stimuli to activate receptor tyrosine kinases that then dimerize and autophosphorylate their catalytic domains. In doing so, SH3-domain containing adaptor proteins facilitate the activation of the membrane-bound GTPase, Ras that then activates effector proteins that signal a downstream kinase cascade that involves Raf kinase, MEK 1/2 and ERK 1/2. ERK 1/2 activation induces nuclear translocation of the activator protein (AP)-1 family of transcription factors that target pro-proliferative genes such as cyclins, growth factors, and cytokines. This response must be tempered as too much activation of this proliferative pathway may lead to overgrowth and cancer.

Other types of MAPKs that are stimulated under stress are JNK and p38. One major difference, however, is that these MAPK proteins are activated directly by factors often deleterious to the cell, such as hyperosmosis, oxidative stress, UV radiation, or inflammatory cytokines. Additionally, p38 and/or JNK

pathways cross-talk with the Ras/Raf/MEK/ERK pathway and act on similar effectors that result in the reparative and proliferative downstream responses. However, p38 and JNK also induce pro-apoptotic proteins. For example, whereas JNK targets the transcription factor c-Jun to activate transcription of genes necessary for proliferation, differentiation, and inflammation, it also upregulates p53, a tumor suppressor and stimulator of apoptosis. Likewise, p38 upregulates pro-apoptotic proteins as well, and it is difficult to distinguish separate effector proteins between the two and depends on the cell context. Overall, their pathways have been shown to regulate apoptosis, inflammation, growth or cell cycle arrest, cell differentiation, and survival.

One important survival pathway that is activated in beta cells is through insulin signaling, where Akt is the major hub of this pathway. Specifically, insulin binds to the insulin receptor, inducing a confirmational change leading to trans-autophosphorylation of tyrosines on the other member of the homodimer and then recruitment of insulin receptor substrate-1 (IRS-1). Phosphorylation/ activation of IRS-1 at its serine 636 site activates phosphoinositide-3-kinase (PI3-K) that subsequently allows for the formation of the second messenger, phosphatidylinositol (3,4,5) trisphosphate (PIP3) which is incorporated into the plasma membrane. PIP3 then forms a complex with Akt where it is phorsphorylated and activated by a phosphoinositide dependent kinase (PDK). Akt then controls cell survival by acting on a number of targets: (1) it binds to Bax and hinders its ability to translocate to the mitochondrial membrane, (2) it activates the mTOR pathway that is responsible activating the translation factor

S6 kinase to initiate ribosomal translation of mRNA, and (3) it phosphorylates and inhibits forkhead box-O1 (FOXO1), a transcription factor that inhibits proliferation.

Overall, these pathways are important cell signaling events that determine the survival of the cell and have been extensively investigated in the field of islet biology in hopes of identifying methods to increase functional beta cell mass. For example, a transgenic mouse model with a constitutively active form of Akt was constructed and found to exhibit increased beta cell proliferation and overall beta cell mass (Fatrai et al. 2006). These pathways have been proven to be of importance within the beta cell and warrant further investigation.

## OXIDATIVE STRESS RESPONSE

Reactive oxygen species (ROS) consist of multiple oxygen-containing radicals that are involved in cell signaling and homeostasis, for example with gene transcription and signaling for host defense mechanisms. ROS are produced by normal cellular metabolism with intermediates that include hydroxyl radical, superoxide radical, or hydrogen peroxide. Chronic accumulation of ROS causes oxidative stress in the islet. Furthermore, diabetic patients and obese patients with diabetes both display reductions in synthesis of glutathione, serum-levels of glutathione peroxidase enzyme and its activity (Sekhar, McKay, et al. 2011, Sekhar, Patel, et al. 2011, Goyal, Singhai, and Faizy 2011). Glutathione is an important anti-oxidant that prevents free radical-induced damage to cellular components. When overexpressed, glutathione peroxidase enzyme protects from

detrimental effects by high ribose concentrations on insulin transcription and insulin secretion (Robertson et al. 2007, Robertson and Harmon 2007).

Normally, beta cells express relatively low levels of anti-oxidant enzymes (Robertson et al. 2003). Although glutathione peroxidase enzyme is absent in islets, manganese superoxide dismutase-1 and -2 (SOD-1 and SOD-2) are the major forms of anti-oxidants that are detected in rodent and human islets (Kang et al. 2014). Without SOD2, high fat-fed mice that have increased oxidative stress, impaired GSIS, but intact insulin sensitivity. Therefore, the increase in oxidative stress within beta cells, in combination with less anti-oxidant capability, directly results in a worsening of beta cell health and the decline in both function and mass, independent of insulin resistance.

**ER STRESS RESPONSE**

The ER is a cellular organelle responsible for calcium storage and protein folding. However, when these processes go awry, such as during the high secretory demand of beta cells in the pre-diabetic state, the ER is subjected to stress and multiple adaptive pathways are stimulated in the unfolded protein response (UPR). The UPR is separated into three arms mediated by inositol-requiring enzyme-1α (IRE1α), activating transcription factor-6 (ATF6), or protein kinase RNA-like ER kinase (PERK). Each of these transducers sense stress in the ER (e.g., increased unfolded or misfolded proteins or inefficient capacity to fold proteins properly) and together facilitate a reduction in protein load, increase

the capacity of the ER to manage the high demand, and trigger cell death if the stress is persistent and unresolved (Hotamisligil 2010).

As briefly mentioned above, the canonical pathway for the UPR arms include: 1) PKR-like endoplasmic reticulum kinase (or PERK) phosphorylates eukaryotic initiation factor 2α, that then upregulates the transcription factors ATF4 and CHOP. Overall, this arm works to suppress general protein translation (Shi et al. 1998); 2) IRE1α activation leads to recruitment of several signaling molecules that can engage survival-related signals, and it's an enzyme with endoribonuclease activity that splices cytoplasmic mRNA, including that for X box–binding protein 1 (Xbp-1), producing the spliced Xbp1-s mRNA that produces an active transcription factor for upregulation of UPR chaperone genes and components of the ER-Associated degradation pathway (Hetz et al. 2011, Lee et al. 2011, Tirasophon, Welihinda, and Kaufman 1998, Wang et al. 1998); 3) Lastly, ATF6 translocates from the ER to the Golgi apparatus, where it is processed by proteases to become an active transcription factor that aids in producing more chaperones to increase the folding capacity of the ER to resolve the misfolded protein issue (Haze et al. 1999, Engin et al. 2014).

Under chronic or severe UPR activation and ER stress, the intrinsic apoptotic pathway and death signals are initiated in beta cells. For example, both ATF6 and PERK increase ATF4 and the transcription factors CHOP and p53 (Hetz et al. 2011). These transcription factors then downregulate Bcl-2 expression, an anti-apoptotic mitochondrial BH3-domain protein, while at the same time upregulate the pro-apoptotic mitochondrial proteins Bax and Bak,

which, as mentioned previously, form a pore on the mitochondrial membrane to release cytochrome c and ultimately induce apoptosis (McCullough et al. 2001, Puthalakath et al. 2007, Galehdar et al. 2010). Additionally, the IRE1 arm of the UPR leads to phosphorylation of ASK1 that then activates JNK, which may also promote Bax/Bak pore formation and apoptosis (Mauro et al. 2006, Hetz et al. 2011). In other words multiple signaling cascades feed into the ER stress-mediated apoptotic program.

Although the events leading to apoptosis are not fully understood, CHOP and phosphorylated JNK are the downstream mediators of the UPR implicated to activate apoptosis through transcriptional upregulation of pro-apoptotic BH3-domain proteins, such as Bim, PUMA, and NOXA and the promotion of Bax/Bak pore complex formation on the mitochondrial membrane (Back et al. , Hetz et al. 2011, Puthalakath et al. 2007). Under such circumstances, apoptosis is triggered by the subsequent release of cytochrome c, which along with caspase 9 and Apaf-1 form the apoptosome, thereby activating executioner caspases such as caspase 3 and 7 (Dahmer 2005).

Aside from initiating the apoptotic pathway, prolonged ER stress inhibits Akt cell survival signaling (Chien-Hung Chen 2011). Recent evidence established that constitutively active Akt inhibits thapsigargin- and tunicamycin-induced phosphorylation of JNK that subsequently leads to less caspase-3 cleavage and death. One proposed mechanism by which this occurs is through activation of phosphatases that directly dephosphorylates Akt to inactivate cell survival signaling.

Pancreatic islets of patients with T2D display increased markers of ER stress, including phosphorylated eIF2α and CHOP (Laybutt et al. 2007, Kim, Xu, and Reed 2008, Huang et al. 2007). Furthermore, diabetic obese db/db mice display increased phosphorylated eIF2α, IRE1α, and Xbp-1 processing. High fat diet and glucolipotoxicity induce ER stress *in vivo* and *in vitro*, respectively, and are currently used as T2D models to examine molecular pathways involved in beta cell death. Free fatty acids evoke the UPR by incompletely understood mechanisms, but ultimately lead to beta cell apoptosis (Tabas and Ron 2011, Ron and Walter 2007). Therefore, it is necessary to understand the ER stress mechanisms in these models of T2D and how p21 is involved in this process.

## I.E. Cell cycle regulation during stress

The cell cycle machinery regulates proliferation and is composed of cyclins that directly interact with cyclin-dependent kinases (CDKs) to allow progression past each cell cycle checkpoint when activated by phosphorylation. CDKs phosphorylate substrates necessary for preparation of the cell to undergo DNA replication and cell mitosis. On the other hand, cell cycle inhibitors (CIPs) bind to and inactivate CDK-cyclin complexes and are divided into two families – the CIP/KIP and INK4 subfamilies (Fuster et al. 2010). The INK4 family of inhibitors (p15, p16, p18, p19) primarily regulates the G1 to S phase transition, whereas the CIP/KIPs (p21, p27, and p57) are capable of regulating entry into each of the phases, G1, S, G2, and mitosis.

The cell cycle machinery is tightly controled under stress conditions in the beta cell. In doing so, there is a mechanism to halt proliferation of damaged cells to allow enough time for repair mechanisms to take place. This process is engaged in order to prevent damaged and dysfunctional beta cells from replicating, thereby preventing the propagation of dysfunctional cells, which, if not restrained, could release aberrant proteins that could serve as auto-antigens.

Expression of the cell cycle regulators is dynamically regulated during a variety of beta cell stresses. For example, upon exposure of beta cells to a high glucose environment, there is an initial stage of proliferation induced and attributed to an increase in cyclins D1 and B1 (Hsu et al. 2009). However, with chronic high glucose, p21 protein levels are induced, suggesting it is activated in response to a chronic stress within the beta cell, presumably to inhibit proliferation (Zhang, Li, et al. 2014). In contrast, exposure to high lipids inhibits glucose-induced proliferation by inducing the cell cycle inhibitors p16 and p18 (Pascoe et al. 2012). Similarly, high circulating fatty acids upregulate the expression of p16 and p18. Furthermore, knockdown of p16 and p18 restores glucose-induced proliferation in beta cells. A later study by this group also determined that within one week of high fat feeding in mice, islets had increased BrdU-positive proliferating cells, while at the same time elevated levels of cyclins D2 and A2. These studies are examples showing that cell cycle regulators are modulated under various stress conditions.

## I.F. p21 and its regulation of functional beta cell mass

p21 is one of the cell cycle inhibitors that is elevated in diabetic models. For example, hyperglycemic Zucker diabetic fatty rats and ob/ob mice display increased p21 expression in islets (Kaneto, Kajimoto, Fujitani, et al. 1999, Kaneto, Kajimoto, Miyagawa, et al. 1999, Keller et al. 2008). Interestingly, pancreatic beta cells of patients with T2D exhibit lower DNA methylation of the p21 gene, and thus higher p21 gene expression (Marselli et al. 2010, Taneera et al. 2013, Dayeh et al. 2014). These data support the idea that p21 regulates functional beta cell mass during stressful environments of the diabetic milieu. It is possible that the increased p21 expression under diabetic conditions contributes to negative regulation on both beta cell mass and beta cell function.

p21 is a CIP that is upregulated during DNA damage and cell stress through both p53-dependent and -independent mechanisms (Russo et al. 1995, Gudas et al. 1995, Michieli et al. 1994, el-Deiry et al. 1994). Findings in my thesis and by others demonstrate that p21 is increased under conditions of endoplasmic reticulum (ER) stress and impairs proliferation (Yamada et al. 2006). p21 halts proliferation at the G1/S transitions by binding to the CDK4/6-Cyclin D and CDK2-Cyclin E complexes. In addition, p21 blocks the G2/M checkpoint by binding to the CDK1-Cyclin B complex. Further functions of p21 include binding to and inhibiting proliferating cell nuclear antigen, which is required for DNA polymerase activity (Soria et al. 2008). In fact, p21 knockout mice were examined to determine whether islet hyperplasia could be observed, and to further enhance beta cell proliferation during mitogen-induced beta cell

mass expansion by placental lactogen (Cozar-Castellano, Weinstock, et al. 2006). However, their findings concluded that p21 did not influence any of these factors, and compensation by other cell cycle inhibitors could be the reason for the fact that p21 alone is not essential for maintaining beta cell cycle arrest.

Although the role for p21 during stress has been primarily implicated to halt proliferation for cell repair, some evidence suggests that it is involved in the death process. Transgenic mice with beta-cell specific p21 overexpression in adulthood displayed hyperglycemia and increased apoptotic cells over time (Yang et al. 2009). Furthermore, human islet amyloid polypeptide (IAPP), a hormone co-secreted with insulin, acts as another stressor of beta cells and induces p21 expression and apoptosis (Zhang et al. 1999, Zhang, Fujita, and Tsuruo 1999). Lastly, both oxidative stress and chronic elevated glucose also induce p21 expression (Zhang, Li, et al. 2014, Kaneto, Kajimoto, Fujitani, et al. 1999). The mechanisms by which p21 induces death are still unclear, however, beta cells that have suppressed expression of protein kinase C (PKC)-delta have elevated levels of p21 and increased TUNEL+-stained apoptotic cells (Ranta et al. 2011). This suggests that PKC-delta is potentially a way that p21 expression is regulated to induce death. Taken together, p21 is induced by beta cell stressors that lead to a decline in beta cell mass.

Aside from regulating processes that affect beta cell mass, p21 has also been implicated in regulating beta cell function. For example, direct oxidative stress by peroxide, or rat islets with impaired anti-oxidant defenses, exhibit elevated p21 expression that is implicated in the decline of beta cell dysfunction

as it decreases insulin gene transcription when overexpressed in isolated rat islets (Kaneto, Kajimoto, Fujitani, et al. 1999, Tarry-Adkins et al. 2009). Recent findings also show that p21 overexpression in beta cells impaired GSIS, and interestingly increased glucagon secretion from alpha cells (Dayeh et al. 2014). Glucagon negatively regulates beta cell's function of secreting insulin, therefore, this is another way in which p21 regulates beta cell function. However, it is yet to be determined whether p21 induction is evident in all cell types of islets from diabetic donors, or whether its expression regulation is specific to the beta cell.

Findings from the body of work mentioned above indicate that p21 is also transcriptionally upregulated during ER stress, and implicate p21 itself as a pro-apoptotic stimulator in beta cells. However, the molecular events by which p21 induces apoptosis are unknown and further explored in this thesis project. In doing so, it will provide insight to the influence of p21 on functional beta cell mass during the progression to T2D.

## I.G. Thesis Hypothesis & Specific Aims

The overall objective of this thesis project was to determine a common factor between multiple beta cell stressors that is detrimental to functional beta cell mass. Theoretically, by inhibiting or therapeutically targeting this common factor, we will preserve or restore functional beta cell mass. As mentioned previously, cell cycle regulators are involved during a variety of forms of beta cell stress and were examined in this thesis project. Because p21 is upregulated during DNA damage and stress in other cell types, I hypothesized that p21 will

antagonize functional beta cell mass during beta cell stress. The specific aims for this project were to: (1) determine how beta cell stressors modulate cell cycle regulators and examine p21's role in cell death, and (2) determine the contribution of p21 to ER stress-mediated beta cell apoptosis and the molecular events that trigger this process after glucolipotoxicity stress.

# Chapter II. Materials and Methods

## II.A. Cell culture and rodent islet isolation

832/13 and 828/33 cell lines, derived from the parental rat insulinoma cell line, INS-1, were maintained in complete RPMI 1640 medium with (L)-glutamine and 11.2 mM glucose supplemented with 50 units/ml penicillin, 50 µg/ml streptomycin, 10 mM HEPES buffer, 10% fetal bovine serum (FBS) and INS-1 supplement as described previously (Hohmeier et al. 2000, Tran et al. 2003). Both of these clonal cell lines have been engineered to stably express human insulin under the control of the cytomegalovirus (CMV) promoter and thus have robust insulin content and GSIS (due to clonal selection); the 828/33 cells also stably overexpress Bcl-2. Hepatocellular carcinoma HepG2 cells were maintained in DMEM containing 4.5 g/L (D)-glucose, 2 mM (L)-glutamine, 50 units/ml penicillin, 50 µg/ml streptomycin, 1 mM sodium pyruvate, and 10% FBS. Pancreatic islets were isolated from male Wistar rats weighing 200-250 g, C57BL6 wild-type mice at 8-10 weeks of age, and F2 hybrid B6129SF2/J wild-type and p21-knockout (B6.129S2-cdkn1; #003263) mice at 8-10 weeks of age (from Jackson Laboratory) using a protocol approved by the Indiana University School of Medicine Institutional Animal Care and Use Committee (Li et al. 2009). Islets were cultured in RPMI medium with (L)-glutamine and supplemented with 8 mM glucose, 10% FBS, 50 units/ml penicillin, and 50 µg/ml streptomycin. Freshly-isolated islets were hand-picked into separate treatment groups and placed in the treatment media on the same day of isolation.

**II.B. Drug treatment groups and adenoviral transduction**

Beta cell lines were treated with complete medium containing the synthetic glucocorticoid dexamethasone (100 nM for 16 h, BioVision, Mountain View, CA) or the sarco/endoplasmic reticulum $Ca^{2+}$ ATPase (SERCA) inhibitor thapsigargin (1 μM for 6 h, Sigma, St. Louis, MO) and compared to DMSO (Sigma) as a control. Isolated primary islets were treated with 100 μM dexamethasone or 1 mM thapsigargin for either 24h (rat islets) or 48h (mouse islets) in complete RPMI medium. Dose- and time-dependent experiments were also performed with thapsigargin treatment. For dose-dependent experiments, 832/13 cells were treated with DMSO or 250, 500, or 1000 nM for 6 h. For time-dependent experiments, 832/13 cells were treated with DMSO or 1000 nM thapsigargin for 0.5, 1, 2, 4, or 6 h. At the end of the experiments, cells were harvested for collection of either mRNA for either quantitative real-time PCR or protein for immunoblotting.

For cytokine experiments, beta cell lines were treated with complete medium containing 10 ng/mL recombinant rat IL-1β and 100 U/mL recombinant rat IFNγ (Prospec, Brunswick, NJ) that were each reconstituted in 0.1% BSA made in water. Cells were treated with the combination of both cytokines for either 6 or 24 h and later underwent cytoxicity and viability assays to measure nitric oxide production or the nitrate/nitrate ratio using the Griess assay (Promega G2930).

Beta cell lines were treated with complete medium containing BSA vehicle or BSA-conjugated palmitate (400 μM for 2, 4, 6, or 8 h) as previously described

(Chen et al. 2010) in either 5 mM glucose- or 20 mM glucose-containing complete medium. In short, BSA-conjugated palmitate was prepared by dissolving sodium palmitate (Sigma, St. Louis, MO) in a 1:1 ethanol/water solution for 10 min at 65°C making a final stock concentration of 150 mmol/l. The control BSA vehicle was prepared with the 1:1 ethanol/water solution mixed with BSA (10% in water, Sigma, St. Louis, MO). The stock palmitate solution was then complexed with BSA (10% in water) at a 5:1 fatty acid/BSA ratio by incubating it for 1h at 37°C. BSA-conjugated palmitate and the vehicle-BSA solutions were aliquoted and frozen in -20°C until ready to treat cells. These aliquots only underwent a maximum of three freeze-thaw cycles.

For gene overexpression, 832/13 cells were transduced for 48 h with purified adenoviruses expressing green fluorescent protein under the control of the CMV promoter (AdCMV-GFP), human p21 under control of the CMV promoter (AdCMV-p21, Vector Biolabs), or human Bcl-2 under the control of the CMV promoter (AdCMV-Bcl2, kind gift from Dr. Curiel at Washington University (Bilbao, Contreras, Eckhoff, et al. 1999, Bilbao, Contreras, Gomez-Navarro, et al. 1999, Bilbao, Contreras, Mikheeva, et al. 1999) with 200 multiplicities of infection. The exception to this time frame was when using purified adenovirus expressing human dominant negative c-jun N-terminal kinase (JNK1; Seven Hills Bioreagents, Cincinnati, OH) under control of the CMV promoter. In this case, 832/13 cells were transduced for 16 h and treated with 1 μM thapsigargin for the last 6 h of the transduction period. Rat islets were transduced immediately following isolation for 72 h with 1000 multiplicities of infection (assuming an islet

contains 1000 cells). Cells and islets were cultured in adenoviral-containing media only for the first 24 h and subsequently switched into fresh medium for the remainder of the experiment before harvesting.

The pharmacological inhibitors for JNK and p38, 10 μM SP600125 and 10 μM PD169316 (Sigma, St. Louis, MO), respectively, were replenished in complete media every 8 h during the 48 h transduction period with adenovirus. In other words, adenovirus-containing media with fresh drug replaced the older media every 8 h for the first 24 h. The second 24 h consisted of replacing older media with adenovirus-free media with fresh drug every 8 h. Control treatment was DMSO for these experiments.

### II.C. [$^3$H]-Thymidine incorporation

Proliferation was assessed via [$^3$H] methyl-thymidine incorporation into genomic DNA by incubating 832/13 cells or isolated rat islets in 1 μCi/ml for 4 or 16 h, respectively. Islets that underwent drug treatments or adenovirus transduction were separated into triplicate groups of 30 as previously described (Fueger et al. 2012). Cold 10% trichloroacetic acid was used to precipitate DNA from each sample, and subsequently solubilized with 0.3 N NaOH. The total amount of [$^3$H]-thymidine incorporation was measured using liquid scintillation counting and normalized to cellular protein content.

## II.D. Quantitative real-time polymerase chain reaction

To isolate RNA, 832/13 cells and approximately 50 isolated rat islets were lysed in RLT buffer containing 1% β-mercaptoethanol and harvested using the RNeasy mini or micro kit, respectively (QIAGEN, Valencia, CA). Next, cDNA was synthesized using the High Capacity Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Samples underwent real-time PCR using Taqman Gene Expression Master Mix Reagents and Gene Expression assay probes (**Table 1**; Applied Biosystems), except for transcript analysis of Xbp1-s where a FAM-labeled probe for Xbp1-s was made using the following sequence: 5'-GGCCCAGTTGTCACCTCCCC-3'. The forward and reverse primers used contained the sequences 5'-CTGAGTCCGCAGCAGGT-3' and 5'-TGTCAGAATCCATGGGAAGA-3', respectively. The reaction for this sequence consisted of 1 μM FAM labeled probe, 2X Taqman Master mix, 2.5X cDNA, and 4 μM of each primer. The threshold cycle (CT) method (Livak and Schmittgen 2001) was used to calculate relative quantities of mRNA products; each sample was performed in triplicate and normalized by the CT value of GAPDH, an internal control within each reading.

Table 1. List of Taqman assays used.

| **Name** | **Catalog #** | **Probe label** |
|---|---|---|
| Mouse CHOP | Mm01135937_g1 | FAM |
| Mouse GAPDH | 4352339E-1208041 | VIC |
| Mouse p21 | Mm00432448_m1 | FAM |
| Human GAPDH | Hs02758991_g1 | VIC |
| Human p21 | HS00355782_m1 | FAM |
| Rat GAPDH | 4352338E-1211016 | VIC |
| Rat CHOP | Rn01450526_m1 | FAM |
| Rat p21 | Rn00589996_m1 | FAM |
| Rat Bim | Rn00674175_m1 | FAM |
| Rat p16 | Rn01433231_m1 | FAM |
| Rat p16 | Rn00580664_m1 | FAM |
| Rat p18 | Rn00590868_m1 | FAM |
| Rat p19 | Rn00486943_m1 | FAM |
| Rat p27 | Rn00582195_m1 | FAM |
| Rat p57 | Rn00711097_m1 | FAM |
| Rat Bcl-2 | Rn99999125_m1 | FAM |
| Rat Bax | Rn02532082_g1 | FAM |
| Rat Bak | Rn00587491_m1 | FAM |
| Rat Bad | Rn00575519_m1 | FAM |
| Rat Bclx-L | Rn00437783_m1 | FAM |

## II.E. Flow cytometry

After adenoviral transduction, the media from cultured 832/13 cells were collected and spun at 1900 rpm at 4ºC for 3 min in clear polyethylene tubes. Cultured cells were then washed with PBS and trypsinized until cells detached from the plate. Cells were gently collected and spun together with the apoptotic cells (i.e., those collected from the media) at 1000 rpm for 3 min at 4ºC. Supernatants were removed and cells were washed with PBS, vortexed, and spun at 1900 rpm for 10 min at 4ºC. Duplicate samples were either processed for cell cycle or apoptosis analysis. For cell cycle analysis, samples were resuspended and stained with propidium iodide using the Guava cell cycle reagent (Millipore, Billerica, MA). For apoptosis analysis, samples were resuspended and co-stained with Annexin V-APC (Invitrogen, Carlsbad, CA) and propidium iodide (BioVision) in annexin-binding buffer (BioVision). Cells were sorted on a BD FACSCalibur APC instrument using the CellQuest Pro software (BD Biosciences, San Jose, CA). Cell cycle populations were analyzed with the ModFit LT Application.

## II.F. Immunoblot analysis

Cells or approximately 150 isolated islets were washed and lysed with radioimmunoprecipitation buffer (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) supplemented with protease inhibitors (Santa Cruz Biotechnology, Inc) and phosphatase inhibitor cocktails 2 and 3 (Sigma). Total cellular protein content

was quantified using the BCA assay kit (Pierce, Rockford, IL). Protein samples (30 µg) were heated with NuPage reducing agent and resolved on NuPage 10% Tris-Bis gels (Invitrogen). Proteins were transferred onto Immobulin-FL membranes (Millipore) and membranes were incubated overnight at 4ºC with primary antibodies (see Table 2) that were diluted in polyvinylpyrrolidone (PVP) (Sigma) (Haycock 1993) or Signal Enhancer HIKARI 250 (SE) (Nacalai USA). The loading control for all immunoblots was actin. Primary antibodies were detected by incubating membranes with IRDye 700- or 800-conjugated secondary antibodies for 1 h; fluorophore-labeled protein bands were detected on the Odyssey System (LI-COR, Lincoln, NE) and were quantified using ImageJ software (National Institutes of Health, Bethesda, MD).

Table 2. List of primary antibodies.

| **Antibodies** | **Dilution** | **Catalog #** | **Company** |
|---|---|---|---|
| Actin | 1:5000 PVP | 691001 | MP Biomedical |
| Akt | 1:1000 SE | 2920S | Cell Signaling |
| Bak | 1:1000 SE | 06-536 | Millipore |
| Bax | 1:1000 PVP | 06-499 | Millipore |
| Bcl-2 | 1:1000 SE | sc-7382 | Santa Cruz |
| Bim | 1:1000 PVP | 2933P | Cell Signaling |
| Caspase 3 | 1:1000 PVP | 9662S | Cell Signaling |
| Caspase 8 | 1:1000 PVP | 9746S | Cell Signaling |
| c-Jun | 1:1000 SE | 9165S | Cell Signaling |
| eIF2$\alpha$ | 1:1000 PVP | 2103S | Cell Signaling |
| FOXO1 | 1:1000 SE | 2880S | Cell Signaling |
| IRS-1 | 1:1000 SE | 51-9002004 | BD Transduction Lab |
| JNK | 1:1000 PVP | 9252S | Cell Signaling |
| p21 | 1:200 PVP | sc-397 | Santa Cruz |
| phospho-Akt [T308] | 1:1000 SE | 4056S | Cell Signaling |
| phospho-cdk1 [pTpY14/15] | 1:1000 SE | 44-686G | Biosource |
| phospho-c-Jun | 1:1000 SE | 9164S | Cell Signaling |
| phospho-eIF2$\alpha$ | 1:1000 SE | 3398S | Cell Signaling |
| phospho-FOXO1 | 1:1000 SE | 9461S | Cell Signaling |
| phospho-IRS-1 | 1:1000 SE | 2388 | Cell Signaling |
| phospho-JNK | 1:1000 PVP | 9251S | Cell Signaling |

## II.G. siRNA transfection

For siRNA-mediated suppression of Bax and Bak, preannealed siRNA duplexes from Ambion (ID # 49750 and 190465, respectively) were utilized and compared to a scrambled control siRNA as previously described (Collier et al. 2006). 832/13 cells were incubated for 24 h with complete medium containing a final concentration of 0.1 μM siRNA in Dharmafect transfection reagents (ThermoScientific, Waltham, MA). Subsequently, adenovirus transduction occurred for the remaining 48 h of culture before harvesting for protein and mRNA analysis.

Stealth siRNA technology was utilized to suppress Bim expression (Life Technologies). Stealth control (Life Technologies) was used as a negative control that did not affect basal Bim expression. The custom stealth RNA sequence used for Bim was 5'-CGAGGAGGGCGUUUGCAAACGAUUA-3' and was used at a final concentration of 50 nM using Lipofectamine 2000 (Life Technologies) as the transfection reagent in Optim-MEM Reduced Serum Medium (Life Technologies).

## II.H. Glucose-stimulated insulin secretion

832/13 and 828/33 cells underwent static incubation GSIS after transduction with adenovirus for about 45 h. The cells were plated on two 12-well plates designated for low glucose or high glucose incubation and were at 100% confluent before starting the procedure. Cells were pre-incubated for 1 h in 2.5 mM glucose-containing buffer consisting of 10X secretion assay buffer (1.14 M

NaCl, 47 mM KCl, 12 mM $KH_2PO_4$, 11.6 mM $MgSO_4$), 1M HEPES, 0.25 M $CaCl_2$, 35% BSA (0.2%), $NaHCO_3$ (25.5 mM) in water. Cells were then switched to either 2.5 mM glucose (basal) or 15 mM (stimulatory) glucose-containing buffer for 1 h. Media of each sample (in triplicate) was used in an insulin radioimmunoassay with the insulin Coat-a-Count kit (Siemens Healthcare).

### II.I. Cell death assay

Islets were isolated from wild-type or p21-knockout mice and on the same day treated with DMSO or 10 μM thapsigargin in 8 mM glucose RPMI complete media for 48 h. 40 islets were used in the Cell Death Detection ELISA PLUS assay (Roche Diagnostics). In short, islets were washed, lysed, and 20 μl lysate (in duplicate) was incubated with 1:1 anti-histone-biotin/anti-DNA-peroxidase immunoreagent on streptavidin-coated microplate wells for 2 h. As a positive control, DNA-histone-complex was incubated (in duplicate) in extra wells. After a few washes, a colorimetric substrate was incubated for 5-10 min and measured using a spectrophotometer. Enrichment of histone-associated-DNA nucleosomal fragments in the cytoplasm was then calculated for each read and duplicates were then averaged to give a single value per treatment group. Readings from each mouse were then normalized to its own DMSO control to calculate the fold-induction of nucleosome enrichment with thapsigargin.

## II.J. Statistical methods

For analysis between two groups, Student's t-test was used, and differences were considered significant when $p < 0.05$. Comparisons between GFP- and p21-overexpressing groups in the cell lines were performed using a two-tailed Student's t-test; islet experiments were analyzed using a paired two-tailed t-test. One-way analysis of variance (ANOVA) was used in experiments that had three or more groups. Differences within ANOVA were determined using Tukey's post-hoc tests with significance determined when $p < 0.05$. All data are reported as means ± SEM.

# Chapter III. Stress-Induced Upregulation of p21 Activates Apoptosis in Pancreatic Beta Cells.

## III.A. Synopsis

Diabetes manifests from a loss in functional beta cell mass, which is regulated by a dynamic balance of various cellular processes including beta cell growth, proliferation, and death as well as secretory function. The cell cycle machinery comprised of cyclins, kinases, and inhibitors regulate proliferation. However, their involvement during beta cell stress during the development of diabetes is not well understood. Interestingly, in a screen of multiple cell cycle inhibitors, p21 was dramatically upregulated in INS-1-derived 832/13 cells and rodent islets by two pharmacologic inducers of beta cell stress, dexamethasone and thapsigargin. We hypothesized that beta cell stress upregulates p21 to activate the apoptotic pathway and suppress cell survival signaling. To this end, p21 was adenovirally overexpressed in pancreatic rat islets and 832/13 cells. As expected, p21 overexpression resulted in decreased [$^3$H]-thymidine incorporation. Flow cytometry analysis in p21-transduced 832/13 cells verified lower replication as indicated by a decreased cell population in S-phase and a block in G2/M transition. The sub-G0 cell population was higher with p21 overexpression and was attributable to apoptosis, demonstrated by increased annexin-positive stained cells and cleaved caspase 3 protein. p21-mediated caspase 3 cleavage was inhibited by either overexpression of the anti-apoptotic mitochondrial protein Bcl-2 or siRNA-mediated suppression of the pro-apoptotic

proteins Bax and Bak. Therefore, an intact intrinsic apoptotic pathway is central for p21-mediated cell death. In summary, our findings indicate that beta cell apoptosis can be triggered by p21 during stress, thus being a potential target to inhibit for protection of functional beta cell mass.

## III.B. Introduction

Diabetes is a metabolic disease characterized by a decline in functional beta cell mass that is attributed to either impaired insulin secretion, or an imbalance in the proliferation to death ratio of beta cells. In the adult pancreas, beta cell proliferation in islets is minuscule (Butler et al. 2003, Cozar-Castellano, Fiaschi-Taesch, et al. 2006, Kassem et al. 2000, Teta et al. 2005). However, in diseased states there could be a decrease in the already low rate of proliferation in addition to an increase in death of existing beta cells. Progress towards understanding the events that lead to beta cell death or inhibition of proliferative potential are necessary to identify novel therapeutic targets for diabetes. Specifically, mechanisms to inhibit cell death and release the inhibition on proliferation are essential to protect and/or restore functional beta cell mass in the setting of diabetes.

The cellular mechanisms regulating beta cell proliferation have been extensively studied and reviewed (Cozar-Castellano, Weinstock, et al. 2006). p21 is a Cdk inhibitor that is part of the CIP/KIP family subtype and has been suggested to function as a molecular brake during beta cell mass expansion. p21 is upregulated in pregnancy and mitogen stimulation (e.g., HGF and placental

lactogen) to avoid excessive proliferation (Cozar-Castellano, Weinstock, et al. 2006, Hughes and Huang 2011, Liu et al. 1996).

In addition to its role in the beta cell, p21 has been studied in cancer biology and its expression is increased after DNA damage and cell stress (Gudas et al. 1995, Zhan et al. 1995). This rise in p21 occurs to halt proliferation and permit cell repair. However, if a cell becomes too damaged, its ultimate fate becomes apoptosis, a highly regulated form of cell death mediated through two distinct pathways, the extrinsic or intrinsic pathway, that converge upon activation of the effector caspase 3. Similarly, during the pathogenesis of diabetes, increased cellular stress, such as endoplasmic reticulum (ER) stress due to the increased secretory demand placed on the beta cell, could ultimately lead to apoptosis. Whereas p21 clearly plays a major role in halting proliferation in pancreatic beta cells, whether upregulation of p21 in beta cells directly contributes to the apoptotic death pathway in the development of diabetes remains to be determined.

Here, we discovered that p21 is the only cell cycle inhibitor to be dramatically increased with two independent pharmacologic inducers of beta cell stress. The aim of this study was to gain more insight on the importance and function for the p21 upregulation during these processes. To this end, a p21-overexpressing adenovirus was used to characterize the role of p21 on apoptosis and cell survival signaling within rodent beta cell lines and isolated pancreatic islets.

## III.C. Results

### Dexamethasone and thapsigargin suppress proliferation and preferentially increase p21 transcription.

Both dexamethasone and thapsigargin decreased proliferation in 832/13 cells as indicated by a decrease in thymidine incorporation (**Figure 4A**). To determine the cell cycle inhibitor(s) responsible for the decrease in proliferation, a qPCR screen of the CIP/KIP and INK4/Arf family members was performed in 832/13 cells (**Figure 4B**). Unexpectedly, p21 was the only cell cycle inhibitor markedly upregulated by both dexamethasone and thapsigargin. Whereas p27 was significantly increased 2.7-fold with thapsigargin treatment, it was not induced by dexamethasone. These findings were verified in isolated rat islets and resulted in a nearly 3-fold increase in p21 transcripts with overnight treatment of dexamethasone or thapsigargin (**Figure 4C**). Transcripts for p15, p16, and p57 were undetectable in 832/13 cells.

To examine the dose- and time-dependent induction of p21 during the initiation of beta cell stress by thapsigargin, we treated 832/13 cells with increasing concentrations of thapsigargin and over a time course (**Figure 5**). We used cleavage of caspase 3 as a readout of the development of beta cell stress-mediated cell death. The induction of p21 with increasing doses of thapsigargin largely mirrored that of caspase 3 activation/cleavage. Interestingly, the time-dependent induction of p21 by thapsigargin also coincided with the activation/cleavage of caspase 3.

Figure 4.

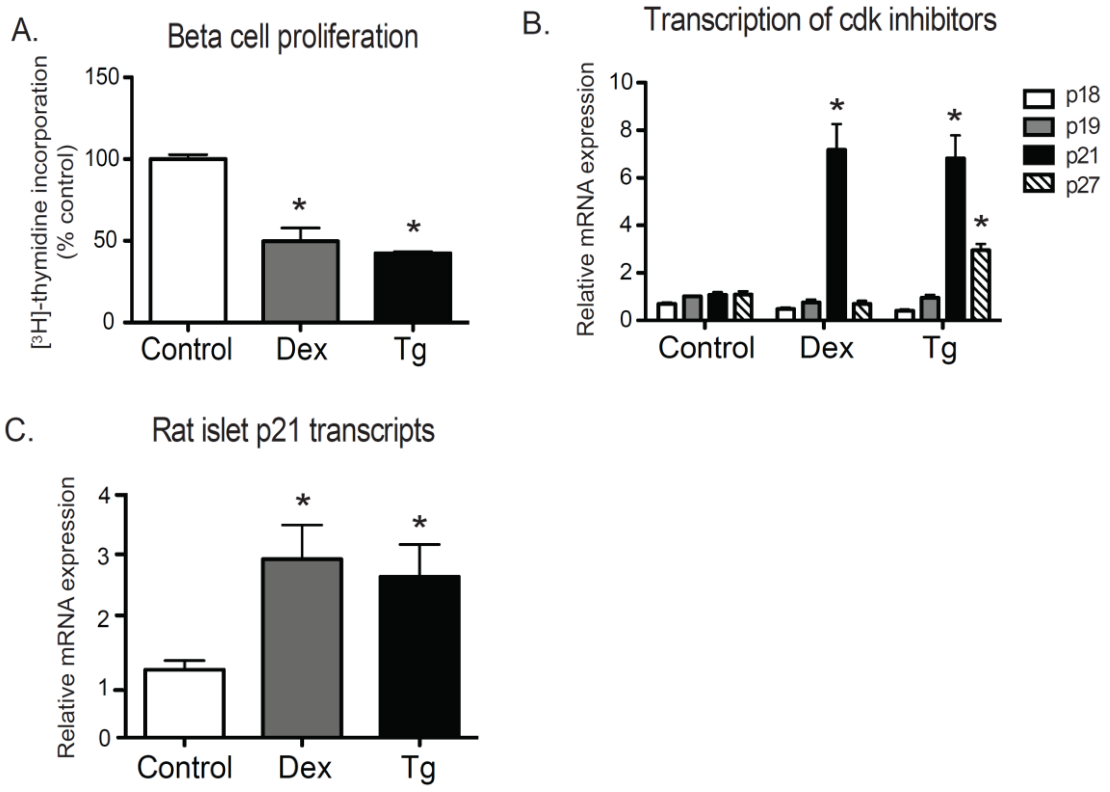


**Figure 4. Dexamethasone and thapsigargin halt proliferation and preferentially increase p21 transcription**. 832/13 cells were treated with the vehicle DMSO (Control), dexamethasone (Dex, 100 nM for 16 h) or thapsigargin (Tg, 1 μM for 6 h). (A) Cell proliferation was assessed by [$^3$H]-thymidine incorporation of triplicate samples, and (B) mRNA levels of cell cycle inhibitors were measured by qPCR. (C) p21 mRNA was measured in isolated rat islets treated overnight with vehicle, 1 μM Dex, or 10 μM Tg. Data are normalized to the DMSO-treated control group and are presented as means ± SEM; n = 3-5. * indicates significance versus Control using a one-way ANOVA test; $p < 0.05$.

**Figure 5.**

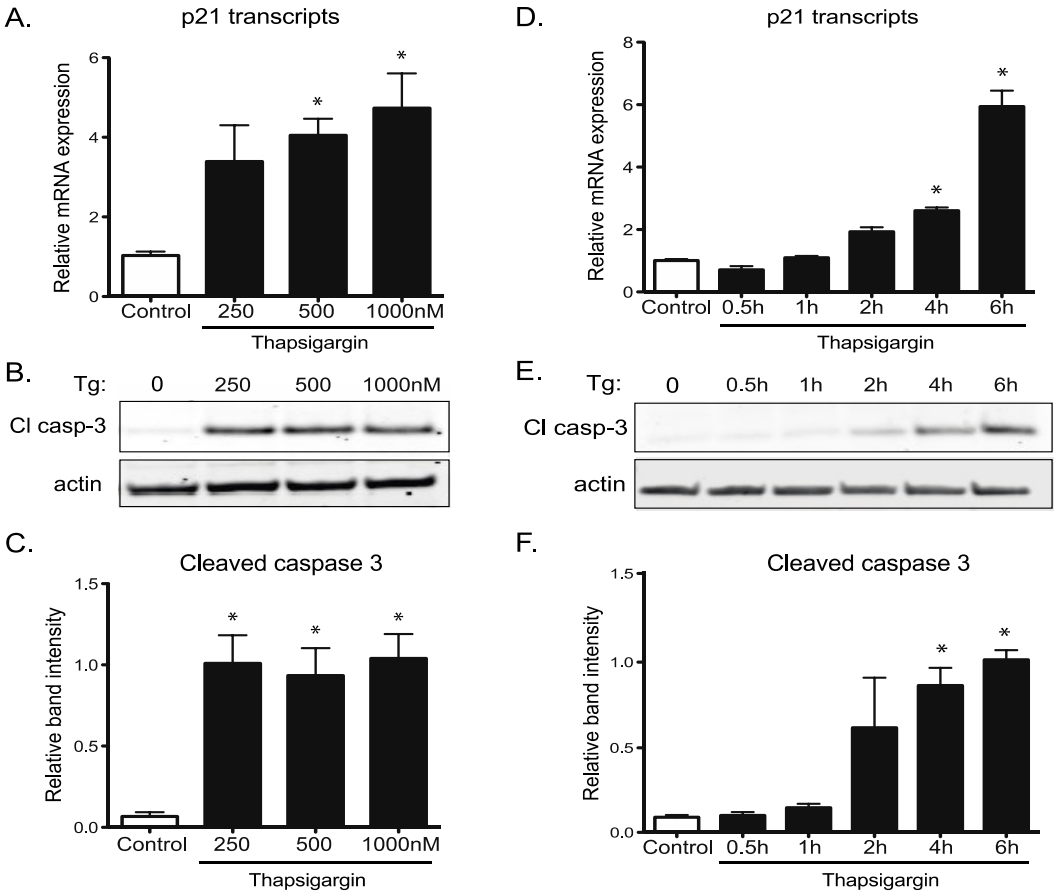


**Figure 5. Dose- and time-dependent upregulation of p21 transcription with thapsigargin**. (A-C) 832/13 cells were treated with the vehicle DMSO (Control) or increasing doses of thapsigargin (250, 500, or 1000 nM) for 6 h. (D-F) 832/13 cells were treated with thapsigargin (1000 nM) for 0.5, 1, 2, 4, or 6 h. (A, D) mRNA expression of p21 transcripts was measured by qPCR and expressed relative to Control. (B, E). Representative western blot of cleaved caspase 3 protein (Cl casp-3) with each treatment and (C, F) quantified results normalized to actin expression. Data are presented as means ± SEM; n = 3-4. * indicates significance versus Control using a one-way ANOVA test; $p < 0.05$.

**Cytokine stress does not induce p21 expression.**

Cytokine stress pathologically contributes to diabetes and the demise of functional beta cell mass. In an attempt to determine whether all forms of stress in beta cells upregulate p21 expression, 832/13 cells were co-treated with two proinflammatory cytokines, IL-1β and IFNγ, both of which cause cytotoxicity in this cell type (Collier et al. 2006). After 6h and 24h of cytokine incubation, p21 expression was not significantly induced as occurs with thapsigargin (**Figure 6**).

**p21 overexpression decreases beta cell proliferation and arrests the cell cycle at G1/S and G2/M transitions.**

To further investigate the role for p21 in beta cells, an adenovirus that overexpressed human p21, thus inhibiting cdk activation (**Figure 7A-B**), was used to transduce 832/13 cells and isolated rat islets. Expression of p21 was adenovirally increased approximately seven- and three-fold in 832/13 cells and rat islets, respectively (832/13 cells: 0.30 ± 0.01 vs. 2.21 ± 0.31 p21/actin, $p < 0.05$; rat islets: 0.86 ± 0.25 vs. 2.34 ± 0.45 p21/actin, $p < 0.05$). In both 832/13 cells and rat islets, p21 overexpression decreased proliferation as indicated by tritiated-thymidine incorporation assays (**Figure 7C-D**). In addition, flow cytometry analysis revealed a significant decrease in the S phase cell population, but an increase in the G2 cell population (**Figure 7E-F**), consequently demonstrating cell cycle arrest at the G1/S and G2/M transitions. Unexpectedly, an increase in the sub-G0 cell population was also evident (**Figure 7G**) and led us to further investigate the role of p21 in apoptosis.

**Figure 6**.

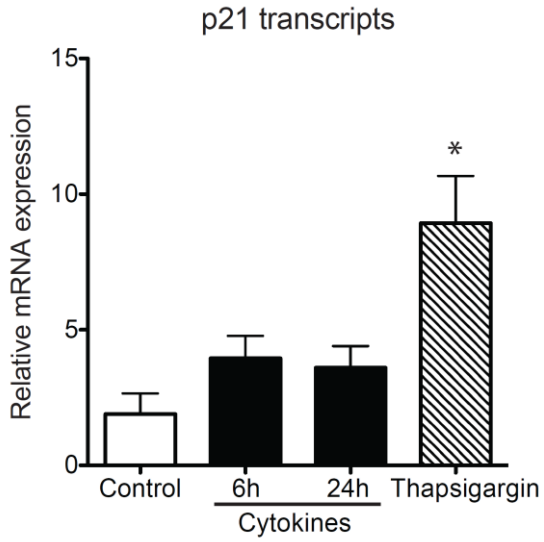


**Figure 6. Proinflammatory cytokines do not induce p21 expression.** 832/13 cells were incubated with IL-1β, TNFα, and IFNγ for 6 or 24 h and compared to incubation with vehicle (Control). As a positive control, cells were incubated with 1uM thapsigargin for 6 h. qPCR was used to measure mRNA expression of p21 transcripts. Data are represented as mean ± SEM, n=4. * indicates significance from control, 6 h cytokines, and 24 h cytokines in a one-way ANOVA using Tukey's post-hoc test.

**Figure 7.**

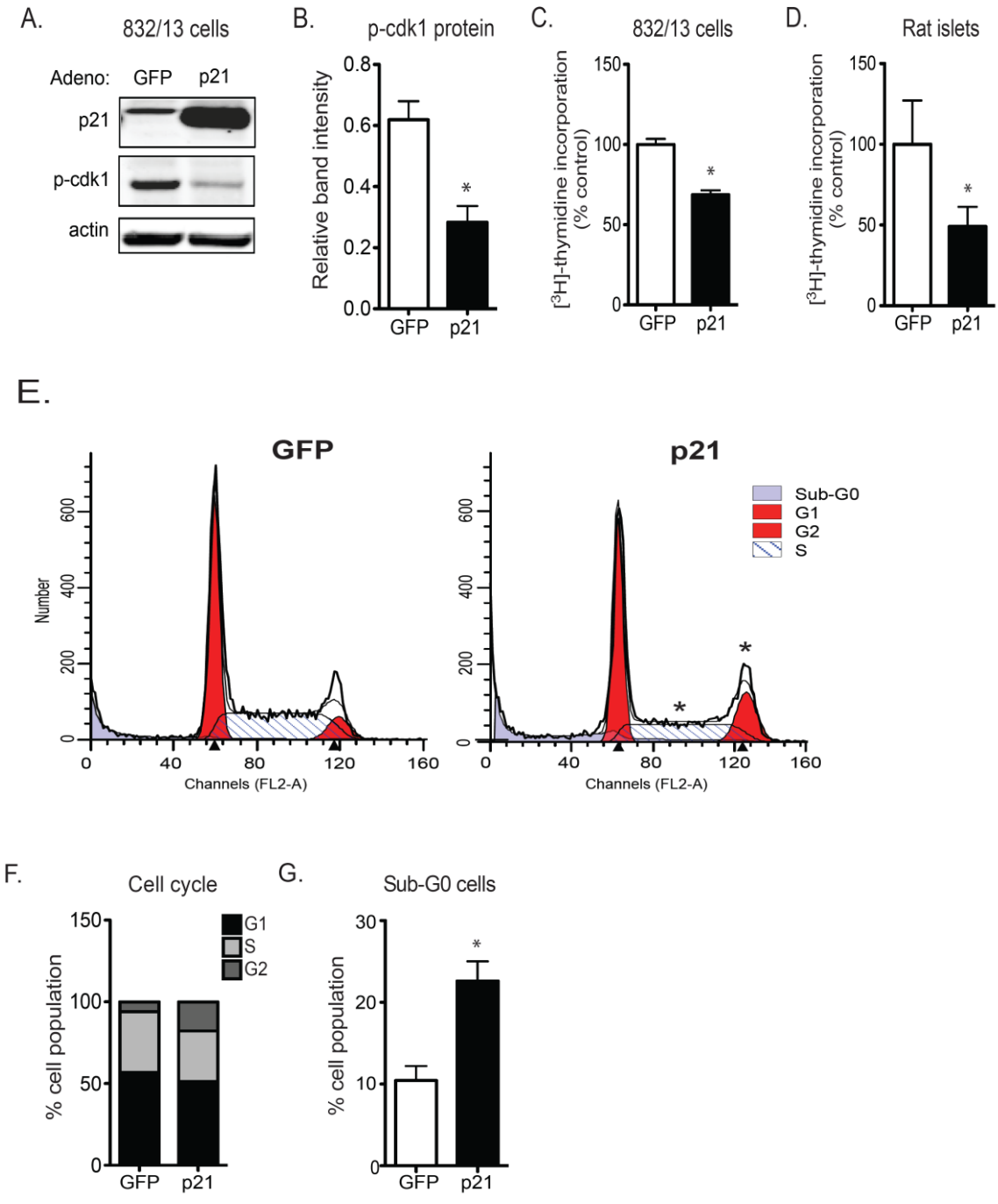


**Figure 7. p21-overexpressing adenovirus inhibits Cdk activation and entry into S and M phase**. (A) Representative western blot of 832/13 cells transduced

with a control GFP- or p21-overexpressing adenovirus and probed for p21 and phosphorylated cyclin dependent kinase 1 (p-cdk1). (B) Quantification of phosphorylated cdk1 normalized to actin. * indicates significance versus GFP; n = 3 independent experiments. (C) 832/13 cells and (D) pooled rat islets of 3 animals were treated with a control GFP- or p21-overexpressing adenovirus and [$^3$H]-thymidine incorporation was measured to assess proliferation. Data are normalized to the control GFP group for [$^3$H]-thymidine incorporation and presented as means ± SEM; n = 3-4 independent experiments in triplicate. * indicates significance versus GFP in an unpaired or paired one-tailed t-test for 832/13 cells and islets, respectively. (E) 832/13 transduced cells underwent flow cytometry and were analyzed by ModFit LT to acquire representative cell cycle peaks for each treatment and (F) to measure the percentage of cells at each cell cycle phase. (G) The percentage of sub-G0 cells were also acquired during this process. Data for cell populations are presented as means ± SEM, n = 4-5 independent experiments with duplicate samples. * indicates significance versus GFP in an unpaired two-tailed t-test, $p < 0.05$.

**p21 directly activates apoptosis in beta cells.**

Using propidium iodide (PI) and annexin co-staining to sort apoptotic cells by flow cytometry (**Figure 8A**), overexpression of p21 was shown to decrease the percentage of viable cells (**Figure 8B**). Additionally, the number of annexin- and PI-positive cells was 7-fold higher with p21 overexpression, indicating a significant increase in the number of apoptotic cells (**Figure 8C**). Furthermore, protein analysis of 832/13 cells transduced with p21-overexpressing adenovirus demonstrated an increase in the hallmark apoptotic marker, cleaved caspase 3 (Cl Casp3) (**Figure 9A, 9C**). More importantly, Cl Casp3 in isolated primary rat islets nearly tripled in p21 overexpression (**Figure 9D, 9F**). To determine whether p21 was disrupting cell survival signaling, levels of phosphorylated Akt (p-Akt) were assessed. Although there was a substantial decrease in p-Akt after p21 overexpression in 832/13 cells (**Figure 9A-B**), this result did not translate to isolated islets under the experimental conditions used (**Figure 9D-E**).

To determine if the induction of p21 during stress and p21's ability to trigger apoptosis were novel phenomena in beta cells, we performed complementary experiments in HepG2 cells, a hepatocyte cell line. Thapsigargin but not dexamethasone induced p21 in HepG2 cells (**Figure 10A**). Interestingly, overexpression of p21 did not stimulate apoptosis in HepG2 cells; as a positive control to establish that HepG2 cells are capable of undergoing apoptosis, HepG2 cells were treated with the topoisomerase inhibitor etoposide, which also induces p21, and this treatment increased Cl Casp3 (**Figure 10B**). Thus, the ability of p21 to induce apoptosis is dependent on the cellular context.

**Figure 8.**

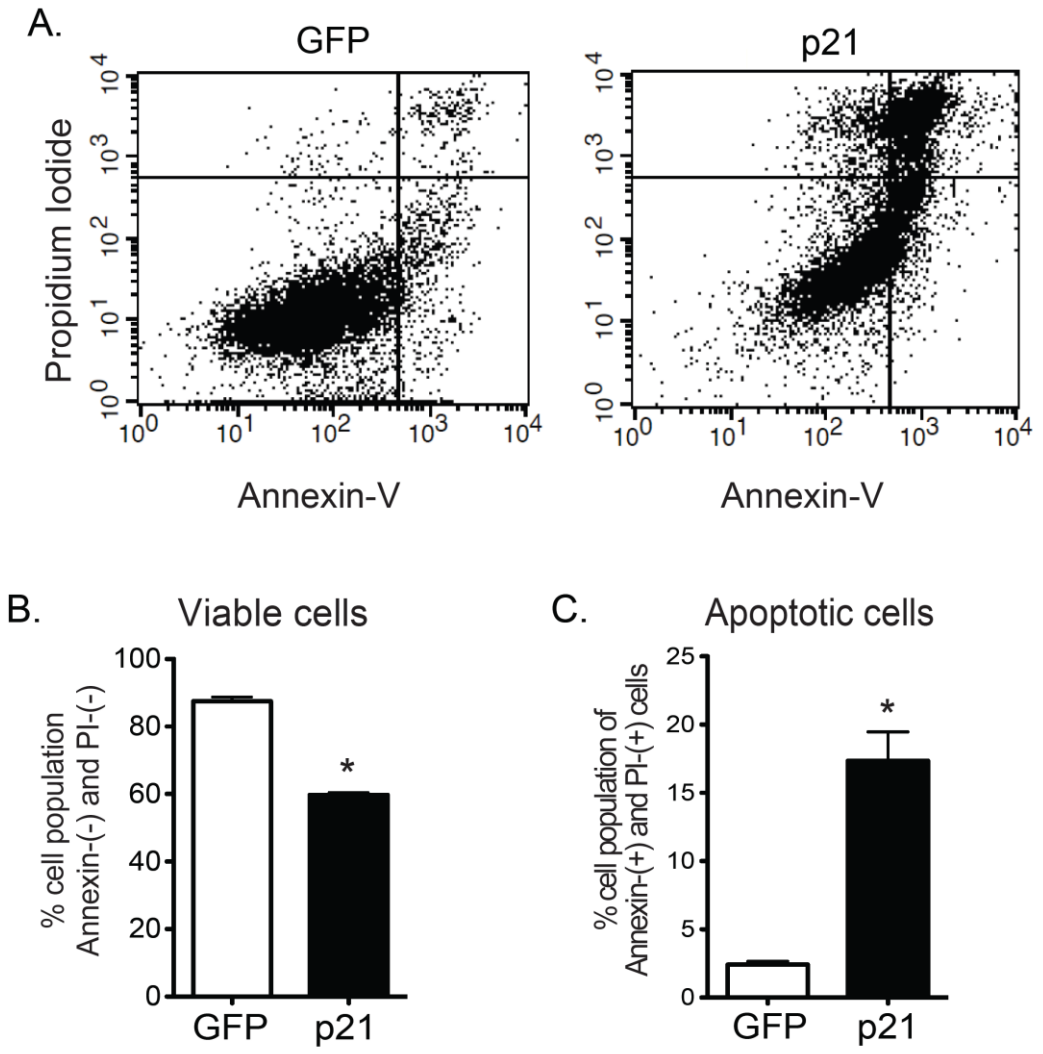


**Figure 8. p21 overexpression increases the number of apoptotic beta cells.** (A) Annexin-V and propidium iodide (PI) were used to co-stain GFP- or p21-transduced 832/13 cells and sorted using flow cytometry. (B) Annexin- and PI-negative cells are represented as viable cells. (C) Annexin- and PI-positive cells are represented as apoptotic cells. Data are presented as means ± SEM; n = 3 independent experiments with duplicate samples. * indicates significance versus GFP in unpaired two-tailed t-tests; $p < 0.05$.

**Figure 9.**

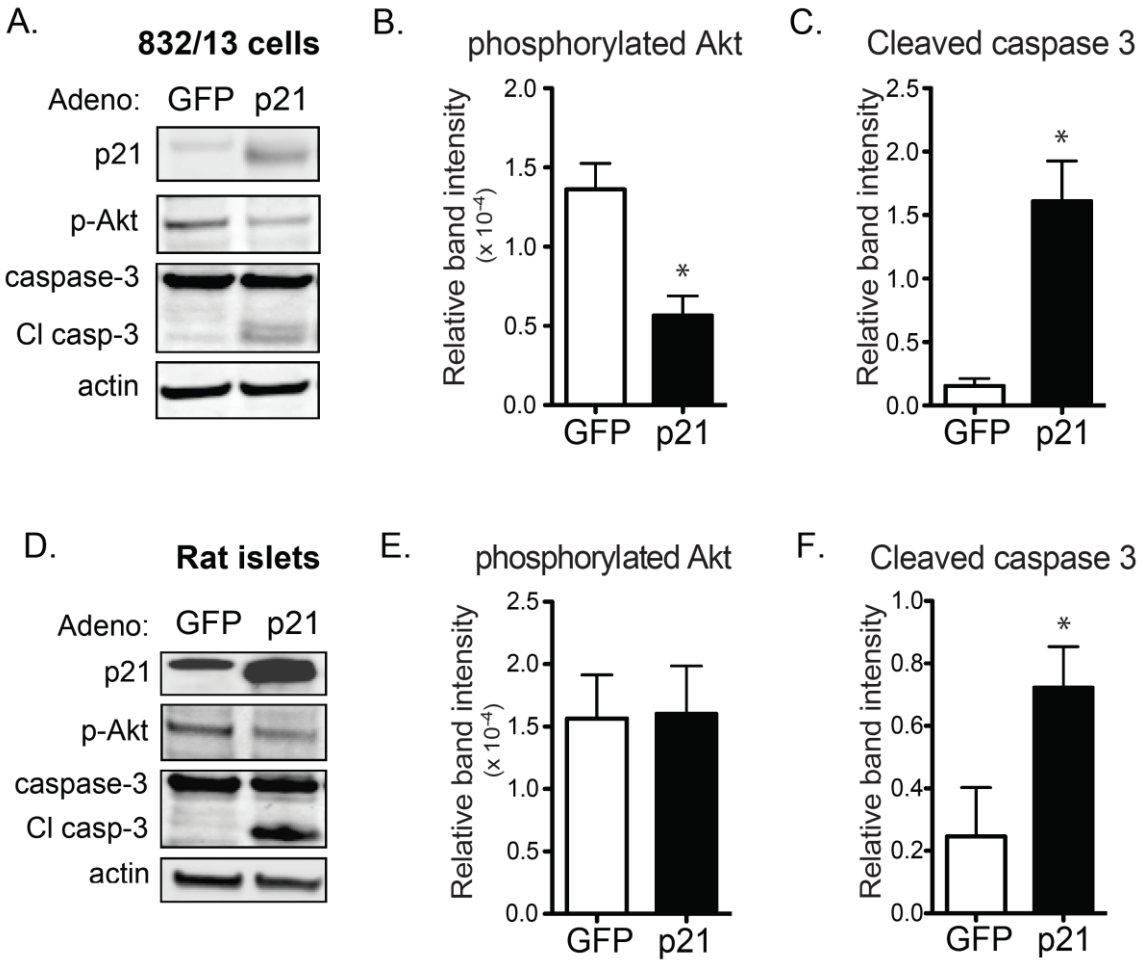


**Figure 9. p21 overexpression activates caspase 3 and decreases cell survival.** Representative western blot images from whole cell lysates of 832/13 cells transduced with GFP- or p21-overexpressing adenovirus for 48 h (A) or rat islets transduced with the same adenoviruses for 72 h (D). Quantification of phospho Akt normalized to total Akt (B, E) and cleaved caspase 3 (Cl casp-3) (C, F), both normalized to actin protein, for lysates from 832/13 cells (B, C) and rat islets (E, F). Data are represented as mean intensities ± SEM, n = 3 experiments. * indicates significance versus GFP in an unpaired two-tailed t-test for 832/13 cells and in a paired two-tailed t-test for islets, $p < 0.05$.

**Figure 10.**

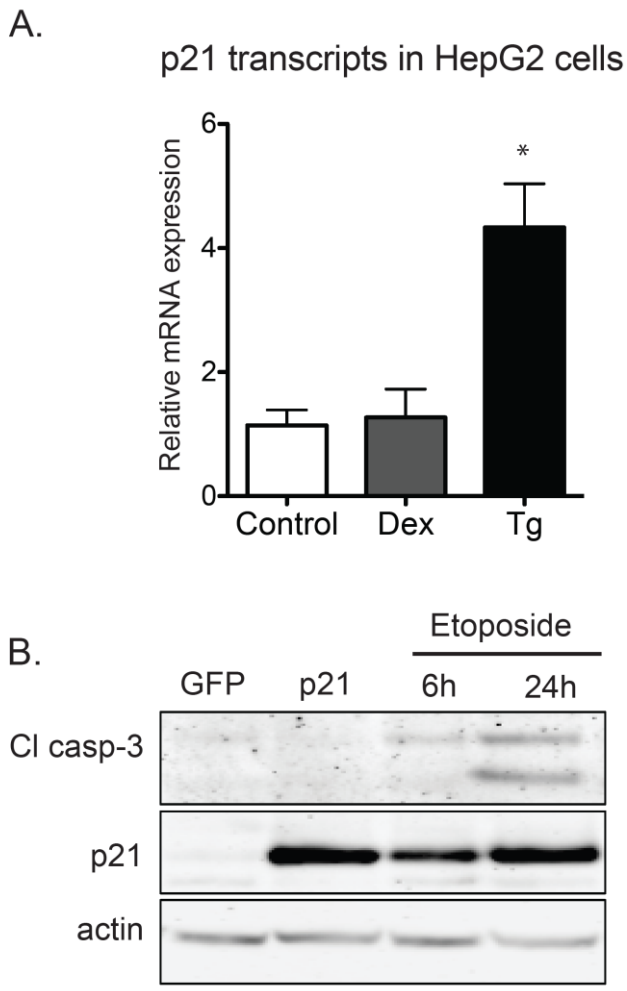


**Figure 10. p21 upregulation does not activate caspase 3 in HepG2 cells.** (A) HepG2 cells were treated with dexamethasone or thapsigargin (1 μM for 24 hr) and mRNA levels for p21 were measured by qPCR. (B) Representative western blot of HepG2 cells were transduced with GFP- or p21-overexpressing adenovirus or treated with etoposide (100μM for 6 or 24 h) as a positive control. Data are normalized to the DMSO-treated control group and are presented as means ± SEM; n = 3-4. * indicates significance versus Control using one-way ANOVA test; $p < 0.05$.

**p21-induced apoptosis is mediated through the intrinsic mitochondrial death pathway.**

The next objective was to determine whether p21 was activating apoptosis through the extrinsic or intrinsic pathway. Protein analysis of caspase 8, an intermediate of the extrinsic pathway, indicated no change with p21 overexpression (**Figure 11A**). mRNA expression of mitochondrial pro- and anti-apoptotic bcl-2 family members that are regulated in the intrinsic pathway were not significantly different with p21 overexpression (**Figure 11B**). However, when utilizing 828/33 cells, a bcl-2 overexpressing beta cell line, p21-mediated caspase 3 cleavage was blocked (**Figure 12A and 12D**). Similarly, thapsigargin-induced caspase 3 cleavage was also blocked in 828/33 cells despite persistent ER stress, as noted by the increase in phosphorylated eIF2α (**Figure 12B and 12E**). Further, acute overexpression of Bcl-2 with a recombinant adenovirus attenuated p21-mediated caspase 3 cleavage (**Figure 12C and 12F**). In addition, siRNA-mediated suppression of the pro-apoptotic Bax and Bak proteins also inhibited p21-mediated cell death as indicated by a decrease in caspase 3 cleavage (**Figure 13**). The promotion of caspase 3 cleavage by p21 was mediated by both Bax and Bak, as siRNA-mediated suppression of either protein significantly reduced caspase 3 cleavage following p21 overexpression, and when both proteins were suppressed simultaneously, there was a further reduction in caspase 3 cleavage. These data suggest that p21-induced apoptosis is mediated through the intrinsic mitochondrial death pathway.

**Figure 11.**

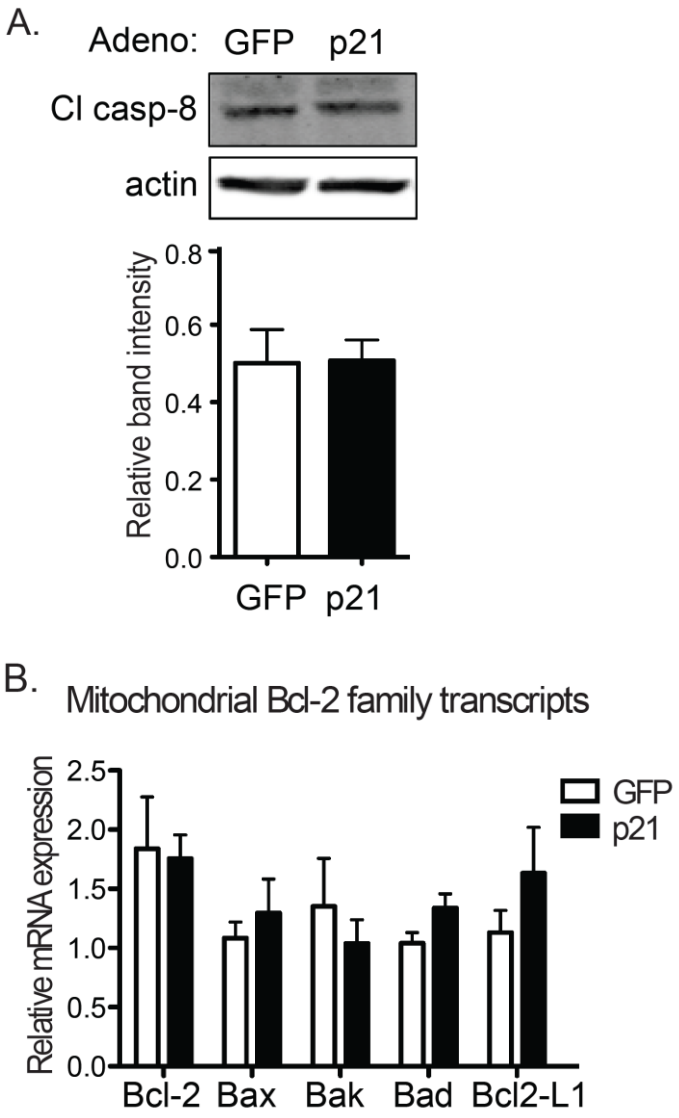


**Figure 11. p21-mediated apoptosis is not regulated through the extrinsic mitochondrial death pathway or by a change in Bcl-2 family member expression.** (A) Western blot analysis of caspase 8 (Cl casp-8) protein levels in whole cell lysates from 832/13 cells transduced with GFP- or p21-overexpressing adenovirus for 48 h. (B) These cells also underwent a qPCR screen of various pro- and anti-apoptotic Bcl-2 family transcripts. n = 3 independent experiments.

**Figure 12.**

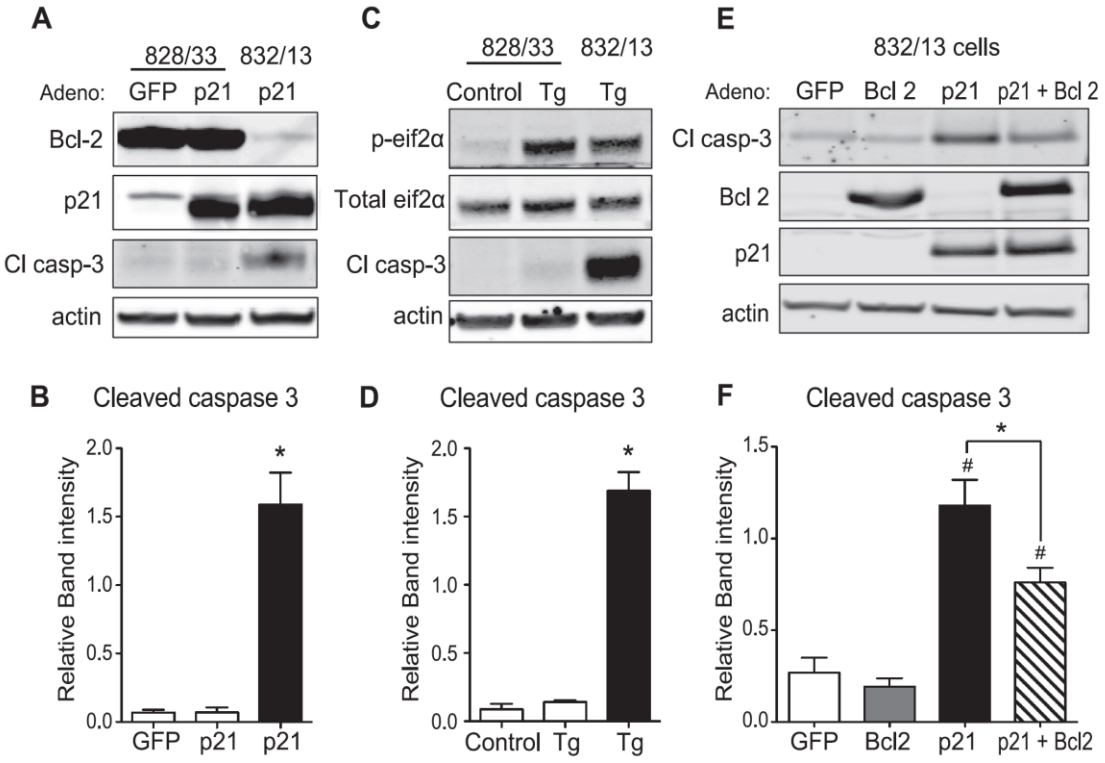


**Figure 12. p21- or ER stress-mediated apoptosis is blocked by Bcl-2 overexpression.** (A) 828/33 cells, which stably overexpress Bcl-2, were transduced with GFP- or p21-overexpressing adenovirus, and as a positive control 832/13 cells were transduced with p21-overexpressing adenovirus. Whole cell lysates underwent western blot analysis for Bcl-2, p21, cleaved caspase 3 (Cl casp-3) and actin as a loading control. (B) Quantification of cleaved caspase 3 normalized to actin. Data are represented as mean intensities ± SEM, n = 3 independent experiments. * indicates significance versus 828/33 cells transduced with GFP- or p21-adenovirus in a one-way ANOVA, $p < 0.05$. (C) Representative western blot of 828/33 and 832/13 cells treated with thapsigargin (Tg) for 6 h and probed for total and phosphorylated eIF2α to indicate ER stress, cleaved caspase 3 (Cl casp-3) to indicate ER stress-induced

apoptosis, and actin as a loading control. (D) Quantification of cleaved caspase 3 normalized to actin. Data are represented as mean intensities ± SEM, n = 3 independent experiments. * indicates significance versus 828/33 cells treated with vehicle or thapsigargin in a one-way ANOVA, $p < 0.05$. (E) 832/13 cells were transduced with GFP-, Bcl-2-, or p21-overexpressing adenovirus for 48 h and caspase 3 activation was observed and (F) quantified via western blot analysis. Data are represented as mean intensities ± SEM, n = 4 independent experiments. * indicates significance between p21- and p21 + Bcl-2-adenovirus-treated groups by ANOVA, $p < 0.05$. # indicates significance versus GFP- and p21-adenovirus-treated groups by ANOVA, $p < 0.05$.

**Figure 13.**

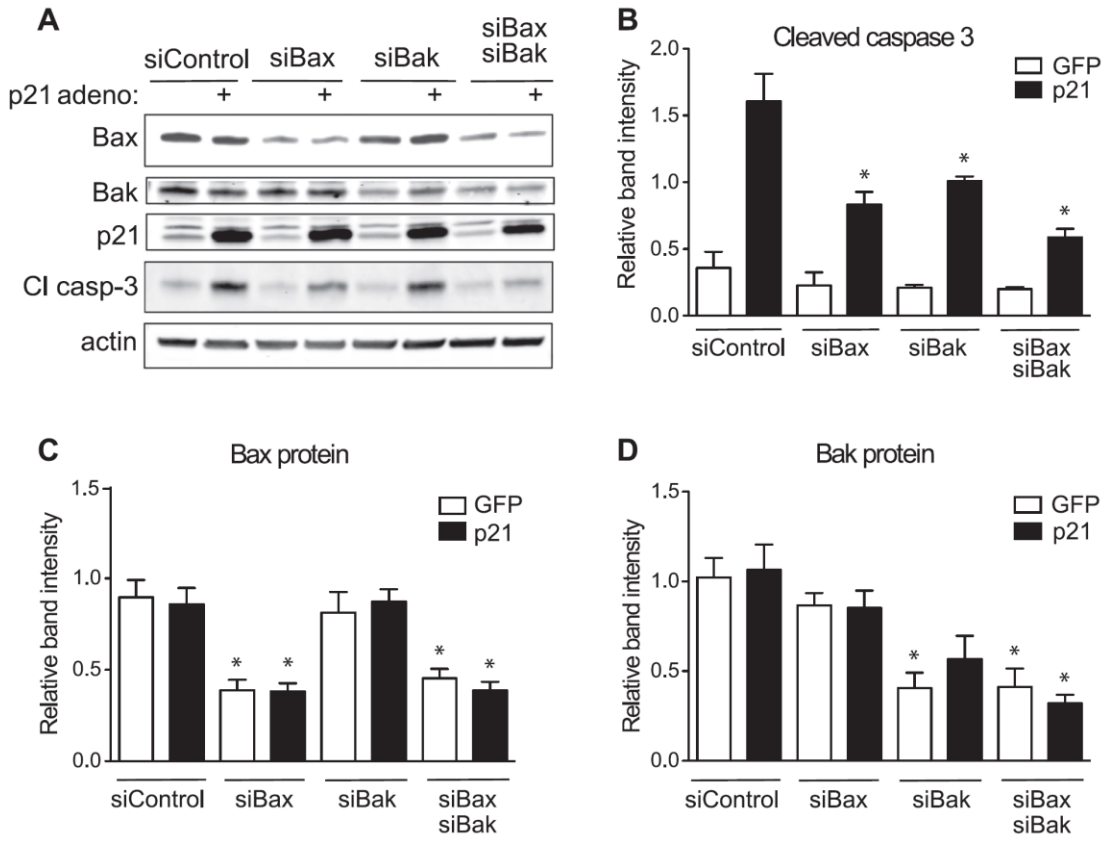


**Figure 13. p21-mediated apoptosis is blocked by siRNA-mediated suppression of Bax and/or Bak.** (A) 832/13 cells were transfected with a scrambled control siRNA (siControl) or siRNAs directed against Bax (siBax), Bak (siBak), or the combination (siBax siBak) for 72 h and transduced with GFP- or p21-overexpressing adenovirus for the last 48 h of transfection for western blot analysis and quantification for cleaved caspase 3 (B, Cl casp 3), Bax (C), and Bak (D) protein normalized to actin. Data are represented as mean intensities ± SEM, n = 4 independent experiments. * indicates significance versus siControl + GFP in a one-way ANOVA test using Tukey's post-hoc, $p < 0.05$.

## III.D. Discussion

During the development of type 2 diabetes, cellular stress impairs beta cell proliferation and function, promotes apoptosis, and ultimately triggers the demise of functional beta cell mass. Therefore, preservation of functional beta cell mass is essential to maintain euglycemia and prevent the transition from glucose intolerance/insulin resistance to frank diabetes. Various stressors known to influence functional beta cell mass during the progression to diabetes include inflammation, ER stress, free fatty acids, and glucotoxicity, to name a few (Donath et al. 2005). However, the precise molecular events linking cellular stress to beta cell impairment and destruction are not fully understood.

In an attempt to determine how a selection of stressors modulates functional beta cell mass and whether independent stressors converge on a uniform pathway, we initially focused on factors regulating cellular proliferation. We thus examined the inhibitory proteins of the cell cycle machinery during exposure to the synthetic glucocorticoid agonist dexamethasone, previously described as a beta cell stressor (Weinhaus et al. 2000), and a pharmacologic inducer of ER stress, thapsigargin. Both dexamethasone and thapsigargin reduced beta cell proliferation, and we speculated that the induction of p21 mediates this response, as it was the only cell cycle inhibitory protein induced by both stressors. Using p21 overexpression in isolated primary rat islets and beta cell lines, we demonstrated that p21 is sufficient to inhibit proliferation by preventing the transition between the G1/S and G2/M phases of the cell cycle. The ability of p21 to prevent cell cycle transitions has been well established given

its ability to directly inhibit the activity of several cyclins and cdks, such as cdk1, necessary for cell cycle progression (Abbas and Dutta 2009).

Several physiological and pathophysiological processes seem to converge on p21 as a mechanism to restrain beta cell growth. Previous work has implicated p21 as a molecular brake for beta cell proliferation during stimulation with mitogens, such as HGF and placental lactogen, to avoid excessive proliferation (Cozar-Castellano, Weinstock, et al. 2006). In addition to mitogen stimulation, endoplasmic reticulum (ER) stress models within beta cells increase p21 levels to inhibit proliferation (Yamada et al. 2006).

Finally, treatment with glucocorticoids, shown here, also induces p21 and presumably limits beta cell proliferation. Although it is counterintuitive that growth factors would induce the expression of a proliferation inhibitor, namely p21, a likely explanation is that these mechanisms are initiated to keep proliferation in check. Therefore, it is possible that negative feedback mechanisms of cell growth, proliferation, and survival signaling pathways are simultaneously activated. This example of negative feedback by a cell cycle inhibitor in the beta cell is not exclusive of p21. Pascoe and colleagues had previously demonstrated that free fatty acids, which themselves can drive beta cell replication, induce both p16 and p18 thereby blocking glucose-stimulated beta cell proliferation (Pascoe et al. 2012). Together, these findings suggest that targeting cell cycle inhibitors may be warranted to maximize beta cell expansion as they serve as molecular brakes to growth-promoting stimuli.

While characterizing the role of p21 in proliferation, sub-G0 cells were notably higher leading us to further investigate p21 in beta cell death. Our findings conclude that p21 initiates apoptosis through the intrinsic apoptotic pathway, and the interplay between pro- and anti-apoptotic mitochondrial proteins are able to modulate the amount of p21-induced caspase 3 cleavage, a hallmark of apoptosis. Few studies have investigated the role of p21 in beta cell death. Interestingly, in a rat model of diabetes, isolated islets of 12-week old Zucker diabetic fatty rats had increased p21 mRNA transcripts that corresponded to a decrease in insulin mRNA (Kaneto, Kajimoto, Fujitani, et al. 1999). Although this study correlated decreased beta cells with an increase in p21, our experiments were able to directly establish that p21 itself is capable of acutely inducing apoptosis through the intrinsic pathway in beta cells, as we demonstrated increased caspase 3 cleavage with p21 overexpression, which could be inhibited by Bcl-2 overexpression or siRNA-mediated suppression of Bax or Bak. In support of our findings, chronically-induced p21-overexpressing mice exhibit hyperglycemia accompanied by increased apoptotic beta cells (Yang et al. 2009). Further, Blandino-Rosano and colleagues demonstrated that when a constitutively active form of Akt was overexpressed in vivo, which robustly increased beta cell replication and induced beta cell apoptosis, as measured by TUNEL staining, deletion of a single p21 allele attenuated the apoptosis (Blandino-Rosano et al. 2012). Thus, chronic elevation of p21 leads to beta cell apoptosis and decreased levels of p21 are able to prevent it. Beyond its level of expression, p21 localization is central to its function as triggering p21 localization

into the cytoplasm allows for its interaction with other molecules that are involved in stimulating apoptosis, such as JNK and even caspase 3 itself (Park et al. 1998, Ranta et al. 2011). Despite its pro-apoptotic function in the beta cell, p21 has had a conflicting role in apoptosis, and it has been suggested to also have anti-apoptotic actions depending on its subcellular localization (Gartel 2005, Koster et al. 2010, Vitiello et al. 2008, Warfel and El-Deiry 2013, Vitiello et al. 2009). An explanation for these contradictory assertions could be that p21's actions are cell type specific. Support for this idea is gained by our observation that p21 overexpression does not trigger apoptosis in a hepatocyte cell line, unlike its ability to do so in beta cells.

One well-known cell survival signaling molecule is protein kinase B/Akt. Here we provide evidence that p21 overexpression inhibits phosphorylation of Akt in 832/13 beta cells. Previous studies have shown that Akt stabilizes p21 protein expression when upregulated and that it is capable of binding to p21 (Blandino-Rosano et al. 2012, Fatrai et al. 2006, Hughes and Huang 2011, Wu et al. 2011, Zhou et al. 2001). However, our findings suggest that p21 is able to negatively modulate Akt, indicating a possible feedback regulation. Further studies are necessary to determine the direct mechanisms for how p21 is able to attenuate Akt activation. Aside from reducing proliferation and cell survival signaling, our findings suggest that p21 is also mediating the death process that occurs as a result of excessive beta cell stress.

In the beta cell, we have determined that p21 is transcriptionally upregulated under stress conditions. p21 overexpression in turn negatively

regulates cell proliferation and survival signaling while simultaneously inducing intrinsic apoptotic pathways in beta cells. Undoubtedly, an increase in p21-mediated apoptosis in the pancreatic islet would be damaging to beta cell function and the integrated insulin secretory capacity. Decreasing the numbers of beta cells during states of insulin resistance or type 2 diabetes, would be detrimental to maintaining glucose homeostasis by compromising the ability to secrete insulin. These findings suggest that inhibiting p21's apoptotic role could be a useful therapeutic target to prevent beta cell death, although experiments to directly test this possibility remain to be completed. Additionally, further studies are necessary to determine the exact mechanism by which p21 induces the death pathway in beta cells. Nevertheless, this work suggests a new mechanism for beta cell destruction during stress.

# Chapter IV. Glucolipotoxicity-induced p21 suppresses pro-survival signaling in pancreatic beta cells

## IV.A. Synopsis

T2D manifests from peripheral insulin resistance and a loss of functional beta cell mass due to decreased beta cell function, survival, or proliferation. The glucolipotoxic environment in the diabetic milieu activates a stress response in beta cells, resulting in decreased survival and programmed cell death through various mechanisms. One key mediator implicated in beta cell dysfunction is FOXO1, a transcription factor phosphorylated and inhibited by the pro-survival kinase Akt. Interestingly, palmitate in combination with high glucose induced expression of p21, a cell cycle inhibitor that activates the intrinsic apoptotic pathway and reduces Akt cell survival signaling. The molecular mechanism by which p21 activates apoptosis in beta cells during metabolic stress is still unknown. We hypothesized that p21-mediated suppression of Akt leads to FOXO1 activation, ultimately resulting in the expression of pro-apoptotic factors. To investigate the molecular events that p21 acts through to increase death and reduce cell survival, we overexpressed p21 with an adenoviral vector in isolated islets and 832/13 cells. p21 overexpression increased phosphorylation of the stress-activated protein kinase JNK. In contrast, FOXO1 phosphorylation was suppressed, indicating activation of FOXO1. Therefore, we propose that p21-mediated inactivation of Akt leads to cell death via JNK and FOXO1 activation. Furthermore, p21 knockout mice were less susceptible to ER-stress induced cell

death. Our findings suggest a molecular mechanism by which metabolic stress- or glucolipotoxicity-induced p21 expression exacerbates beta cell death – a mechanism that could potentially be targeted to protect beta cell mass.

## IV.B. Introduction

Increased intracellular free fatty acids in the presence of hyperglycemia, which is often found in cases of obesity, triggers a pathological process in the beta cells known as glucolipotoxicity. Mechanistically, glucolipotoxicity can induce a variety of processes deleterious to beta cells, including ER stress, inflammation, and oxidative stress. For example, mouse models of glucolipotoxicity such as high fat feeding exhibit increased ROS, inflammatory markers, and hallmarks of ER stress. Because we demonstrated in the previous chapter that p21 is induced by ER stress in beta cells, we speculated that glucolipotoxicity might also induce p21. Evidence from the literature in both human T2D and animal models of diabetes also suggest that p21 may be induced in response to overnutrition and the commensurate stress on the beta cell (Taneera et al. 2013, Keller et al. 2008, Dayeh et al. 2014). The proposed induction of p21 in response to glucolipotoxicity would be likely to abrogate pro-survival signaling, such as Akt signaling. Identifying how pro-survival signaling is compromised with glucolipotoxicity could shed light on pathways that become impaired in T2D, thereby providing novel targets for protecting functional beta cell mass in the context of T2D pathogenesis.

One key mediator implicated in the loss of functional beta cell mass in the form of beta cell dysfunction is FOXO1, a transcription factor that regulates expression of target genes involved in cell death, reactive oxygen species detoxification, DNA repair, and cell cycle arrest (Carter and Brunet 2007). Insulin or growth factor signaling normally activates Akt, which then regulates FOXO1 activity by phosphorylating FOXO1 to inhibit its nuclear localization. In the previous chapter we established that p21 overexpression reduces p-Akt. Therefore, it was hypothesized that p21 suppresses Akt, which then results in activation of FOXO1 in regulating its downstream target gene, Bim, a pro-apoptotic BH3 domain containing protein.

As the previous chapter focused on the induction of the cell cycle inhibitor p21 during ER stress in beta cells, the work here attempted to extend those findings to the clinically relevant scenarios of glucolipotoxicity and T2D. Therefore, the overall aim of the experiments in this chapter will focus on the role of p21 induction during glucolipotoxicity-induced ER stress, and investigation of the cellular pathways that are regulated by p21. As previously noted, overexpression of p21 inhibits Akt activation. Therefore, we propose that p21 is induced by glucolipotoxicity and results in the activation of stress pathways and the reduction of Akt downstream cell survival pathways.

## IV.C. Results

### High fat feeding induces p21.

High fat feeding in C57BL/6 mice has been used as a model for pre-diabetes as it promotes the normal beta cell compensatory responses thought to occur in the pathogenesis of human T2D. If beta cell compensation is prevented such as in the BTBR mouse (Keller et al. 2008) or mice lacking one allele of Pdx1 (Johnson et al. 2003) or both insulin receptor alleles (Kulkarni et al. 1999), mice go on to develop frank T2D. Identifying mechanisms that prevent beta cell compensation are essential to preserving glucose homeostasis in the face of systemic insulin resistance. Thus, we examined the expression of p21 in a microarray experiment with islets from mice fed either a standard chow or 42% high fat diet. Interestingly, p21 was elevated in the islets from high fat fed mice compared to the chow-fed controls (**Figure 14**).

### Glucolipotoxicity induces ER stress, cell death, and p21 expression.

Others have reported that glucolipotoxicity-induced beta cell death is mediated through severe ER stress and is reduced in Bim knockout mice *ex vivo* (Wali et al. 2014). Our previous findings indicate that p21 transcription is induced by ER stress in beta cells. Therefore, we aimed to determine whether glucolipotoxicity-mediated ER stress also upregulates p21. In agreement with previous studies, glucolipotoxicity in beta cells activates ER stress as is evident by the increase in spliced Xbp1 (Xbp1-s) and CHOP expression (**Figure 15A-B**).

Furthermore, cellular stress and death were present after glucolipotoxicity, which resulted in the phosphorylation of the stress kinase c-jun N-terminal kinase (p-JNK) (**Figure 15C, 15D**). Increased cell death was measured by the abundance of cleaved caspase 3, which was significantly increased by 4 h of glucolipotoxicity (**Figure 15D-E**). Importantly, glucolipotoxicity also induced p21 expression (**Figure 15F)**.

**p21 overexpression activates eIF2α but does not activate ER stress**

To further investigate the role of p21 in beta cell stress, an adenovirus that overexpressed human p21 was used to transduce 832/13 cells. Interestingly, p21 overexpression resulted in an increase of p-eIF2α (**Figure 16A-B**). To determine whether or not p21 directly activates ER stress, we also examined the expression of Xbp1-s. Whereas thapsigargin, a chemical inducer of ER stress, dramatically upregulated Xbp1-s (**Figure 16C**), p21 overexpression did not increase Xbp1-s but rather decreased its expression (**Figure 16D**). Therefore, p21 itself does not activate ER stress, but rather ER stress induces p21 expression.

**Figure 14.**

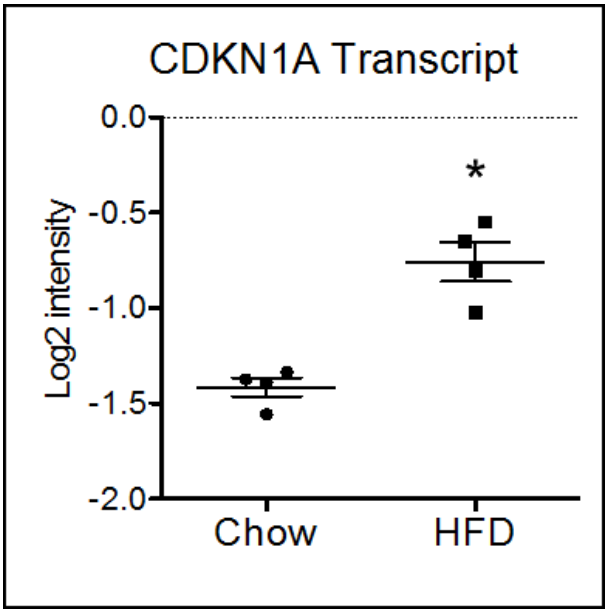


**Figure 14. p21 transcripts are elevated under stress *in vivo*.** Microarray analysis of islets from C57BL/6 mice fed normal chow or 42% high fat diet (HFD) for 16 weeks. $p < 0.05$ ANOVA and Tukey's post-hoc. Data courtesy of Dr. Evans-Molina and Dr. Mirmira laboratories (Sims et al. 2013).

**Figure 15.**

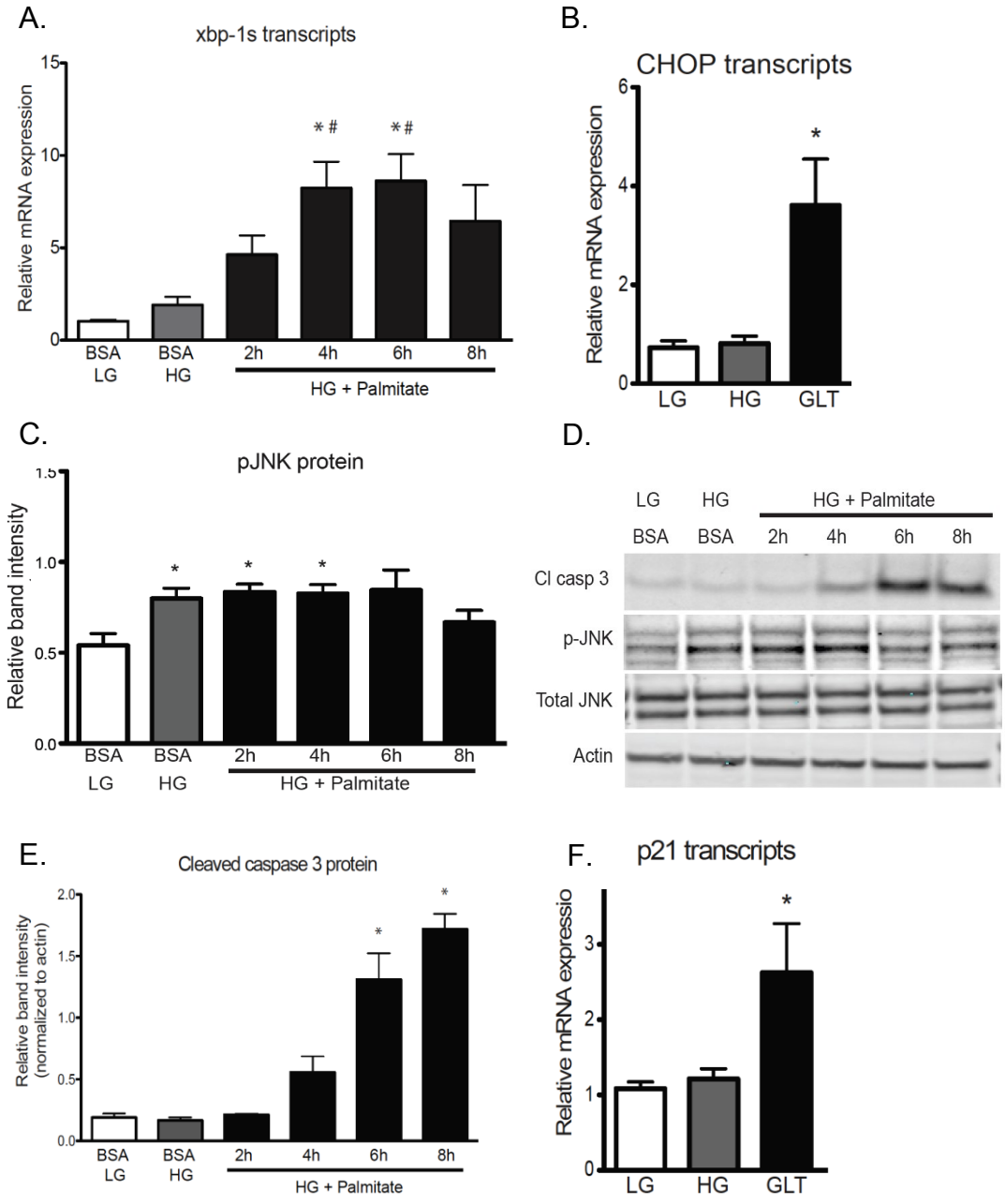


**Figure 15. Glucolipotoxicity activates ER stress, the stress kinase JNK, and p21 expression**. 832/13 cells were treated with 1% BSA in either 5 mM glucose for 8h (LG) or in 20 mM glucose for 8h (HG) and compared to 400 μM palmitate in 20 mM glucose at various time points as stated above or for 2 h (GLT). qPCR was used to measure mRNA expression of (A) spliced Xbp1 (Xbp1-s) and (B)

CHOP. (C) Quantitative analysis of phosphorylated JNK (p-JNK) and a (D) representative western blot of p-JNK and cleaved caspase 3 (Cl casp 3). (E) Quantitative analysis of Cl casp 3 normalized to actin. (F) qPCR was used to quantify p21 gene expression. Data represented as means ± SEM; $n$ = 3-4. *significance vs. LG BSA in one-way ANOVA using Tukey's post hoc; $p < 0.05$.

**Figure 16.**

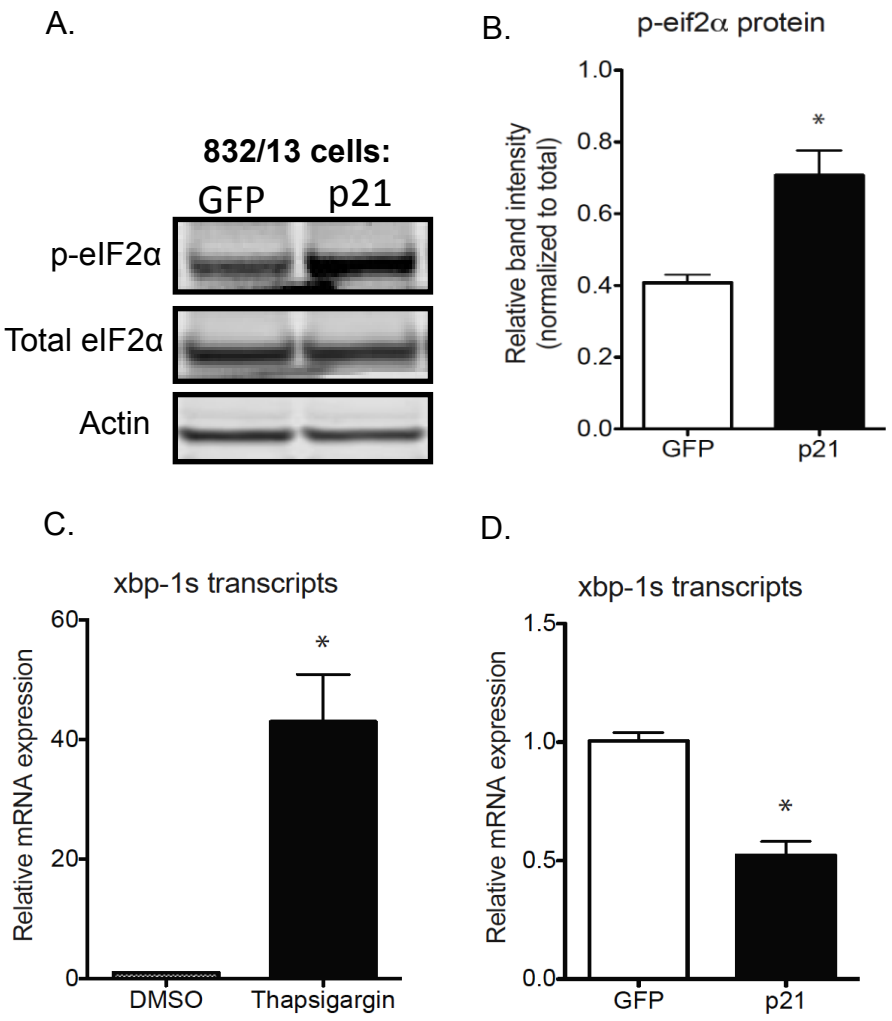


**Figure 16. p21 overexpression activates eIF2α but does not induce ER stress.** 832/13 cells were transduced with an adenovirus overexpressing GFP or p21 for 48 h. Shown are (A) a representative western blot for phosphorylated (p-eIF2α) and total eIF2α (B) and quantified results relative to total eIF2α protein. qPCR was used to measure mRNA levels of spliced Xbp1 (Xbp-1s) in 832/13 cells that were (C) treated with DMSO control or thapsigargin for 6 h, or (D) transduced with GFP- or p21-overexpressing adenovirus.

**p21 overexpression activates JNK preceeding cell death.**

Similar to the observations from glucolipotoxic conditions, p21 overexpression increased p-JNK in isolated mouse islets (**Figure 17A-B**) and 832/13 cells (**Figure 17C**). We next investigated whether p21-mediated caspase 3 cleavage was necessary to activate JNK – i.e., did death beget heightened stress kinase signaling? To test this, we utilized the Bcl-2 overexpressing 828/33 rat beta cell line, which inhibits p21-mediated caspase 3 cleavage (Hernandez et al. 2013). We found that p21 overexpression in this cell line results in similar activation of p-JNK, despite the absence of cleaved caspase 3 (**Figure 17C-17D**). Therefore, we conclude that the activation of this stress responsive kinase occurs before p21-mediated cell death.

**ER-stress induced cell death, but not p21-induced cell death, is dependent on the JNK pathway.**

Previous evidence suggests that the JNK pathway is necessary to induce glucolipotoxicity-induced death and ER-stress mediated deleterious effects on beta cell function (Ozcan et al. 2004, Martinez et al. 2008). To verify these findings, we utilized a dominant negative JNK1 (JNK-DN)-overexpressing adenovirus to inhibit the JNK pathway in 832/13 cells. JNK activity was measured by quantifying the amount of phosphorylated c-Jun (p-c-Jun), a well-known JNK target protein. Upon thapsigargin treatment, JNK-DN overexpression resulted in a decrease of p-c-Jun compared with thapsigargin treatment alone (**Figure 18A**).

Additionally, JNK-DN overexpression blunted thapsigargin-induced caspase-3 cleavage **(Figure 18B)**.

To determine whether activation of JNK is necessary for p21-mediated death, we used a JNK inhibitor, SP600125, to inhibit JNK (**Figure 19A**). Although JNK activity was inhibited, as is evident by the decreased phosphorylation of c-Jun (**Figure 19B**), p21-mediated cell death was not reduced (**Figure 19B-C**). Because multiple stress kinases are activated during stress, we used a p38 MAPK inhibitor in combination with the JNK inhibitor (**Figure 20**). In this case, cell death in response to p21 overexpression persisted and was not inhibited with either the p38 inhibitor, JNK inhibitor, or the combination of the two inhibitors. These data suggest that although p21 activates stress and kinases such as JNK, p21-mediated death is not solely dependent on their downstream signaling.

**Figure 17.**

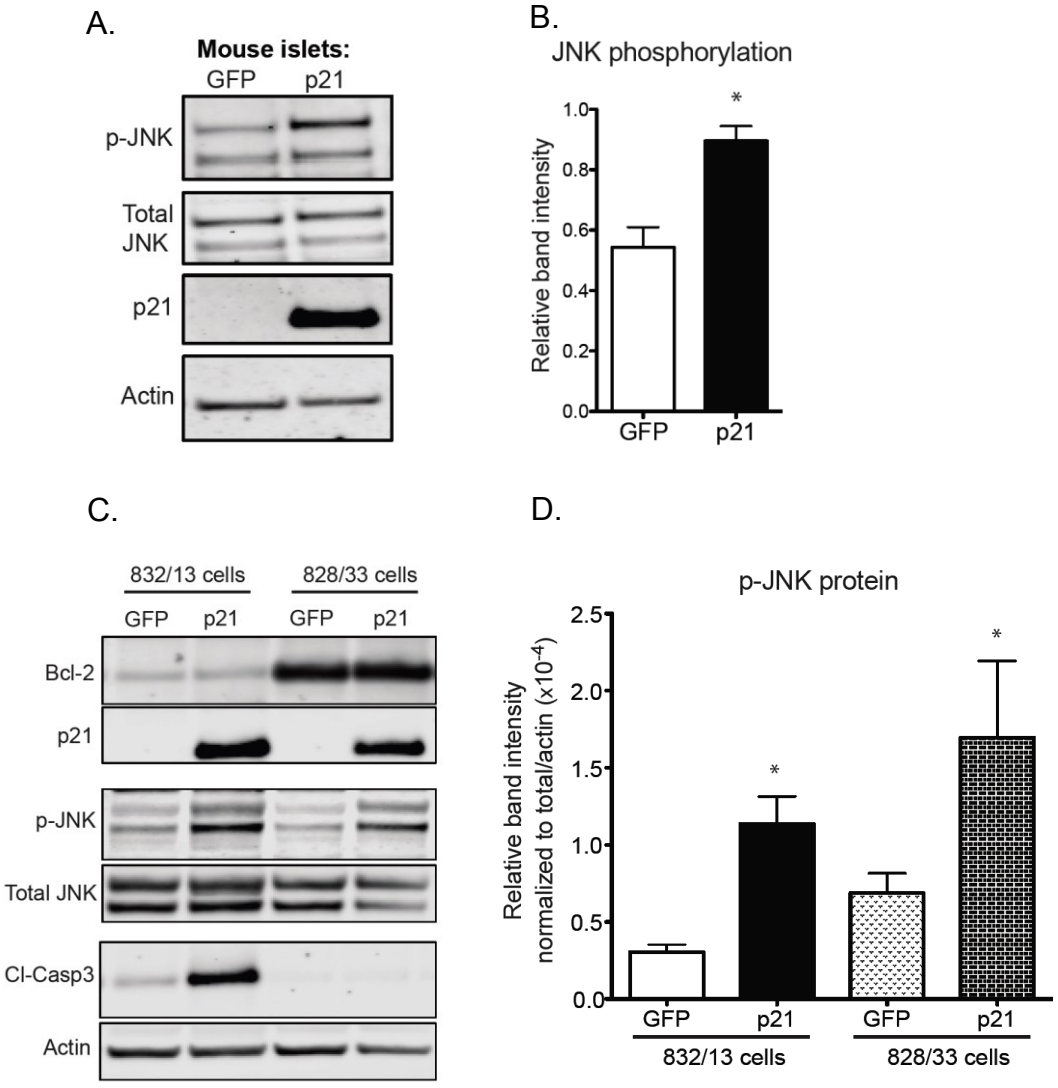


**Figure 17. p21 overexpression induces phosphorylation of JNK prior to cell death.** Shown are a representative Western blot analysis and quantification of p-JNK in GFP- or p21-overexpressing mouse islets (A-B, respectively). (C) 832/13 or 828/33 cells were transduced with adenoviruses for 72 or 48 h, respectively. (D) Quantification of p-JNK was normalized to total JNK/actin. Data are represented as means ± SEM; n=3-4. * indicates significance vs GFP; $p < 0.05$.

**Figure 18.**

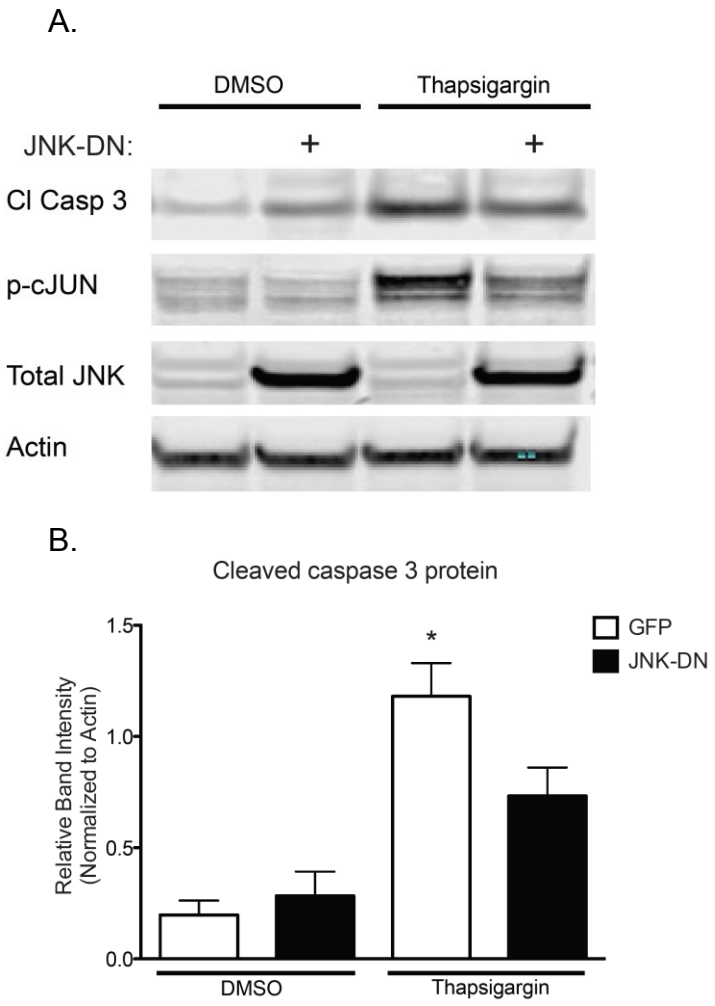


**Figure 18. Dominant negative JNK1 inhibits ER stress-induced cell death.** 832/13 cells were transduced with a GFP- or a dominant negative JNK1 (JNK-DN) for 16 h and underwent 1 μM thapsigargin treatment for the last 6 h. (A) Representative western blot image of cleaved caspase 3 (Cl Casp3), p-cJun, total JNK, and actin as a loading control. (B) Quantification of Cl Casp3 protein. Data represented as mean ± SEM, n=4. * indicates significance vs. DMSO treated GFP-overexpressing cells, # indicates significance vs. Thapsigargin treated GFP-overexpressing cells.

**Figure 19.**

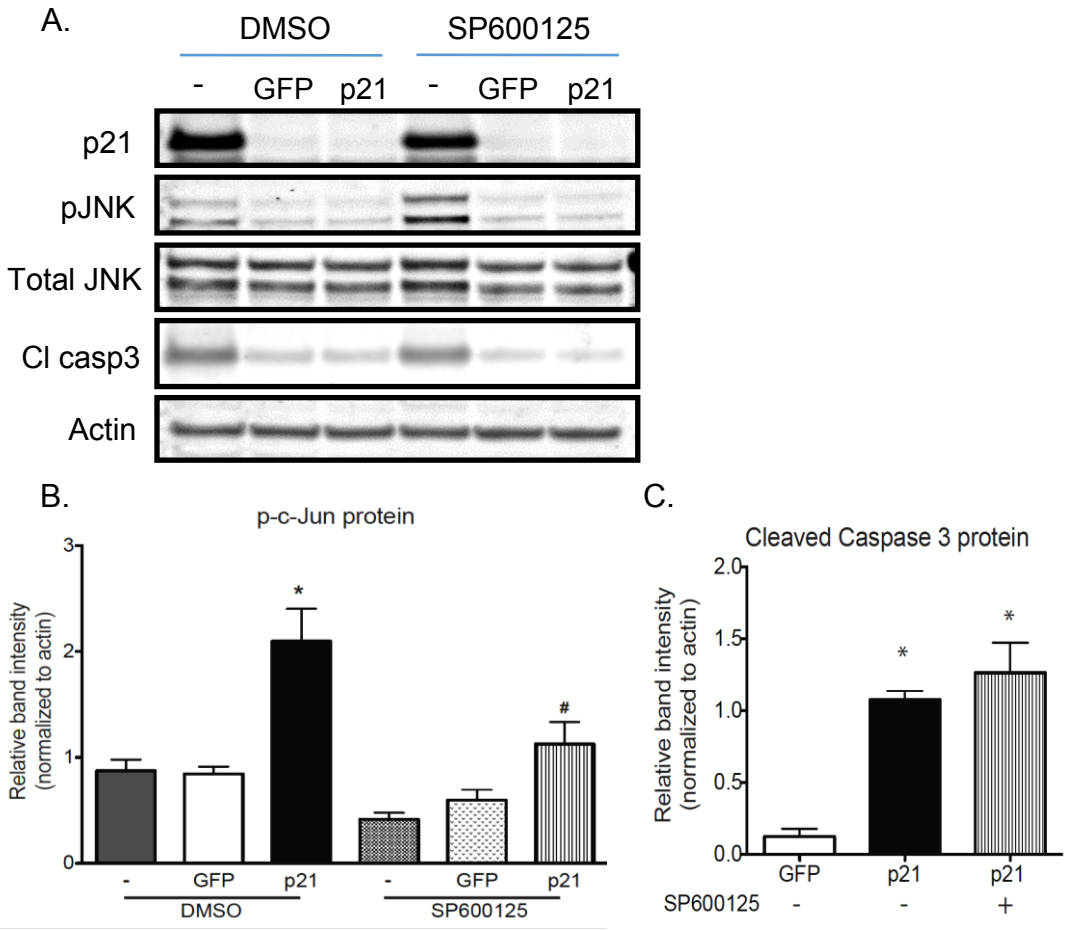


**Figure 19. p21-mediated cell death is not dependent on the JNK pathway**. GFP- or p21-overexpressing adenovirus was used to transduce 832/13 cells for 48 h. At the same time, the JNK inhibitor SP600125 or DMSO were used to treat cells every 8 h during the 48 h transduction period. (A) Representative western blot image of p21, p-JNK, total JNK, cleaved caspase 3 (Cl casp3), and actin. Quantitative analysis of (B) phosphorylated c-Jun (p-c-Jun) protein and (C) cleaved caspase 3 protein. Data are represented as means ± SEM. * indicates significance vs. GFP DMSO and # indicates significance vs. p21 DMSO in a two-way ANOVA using Tukey's post-hoc test.

**Figure 20.**

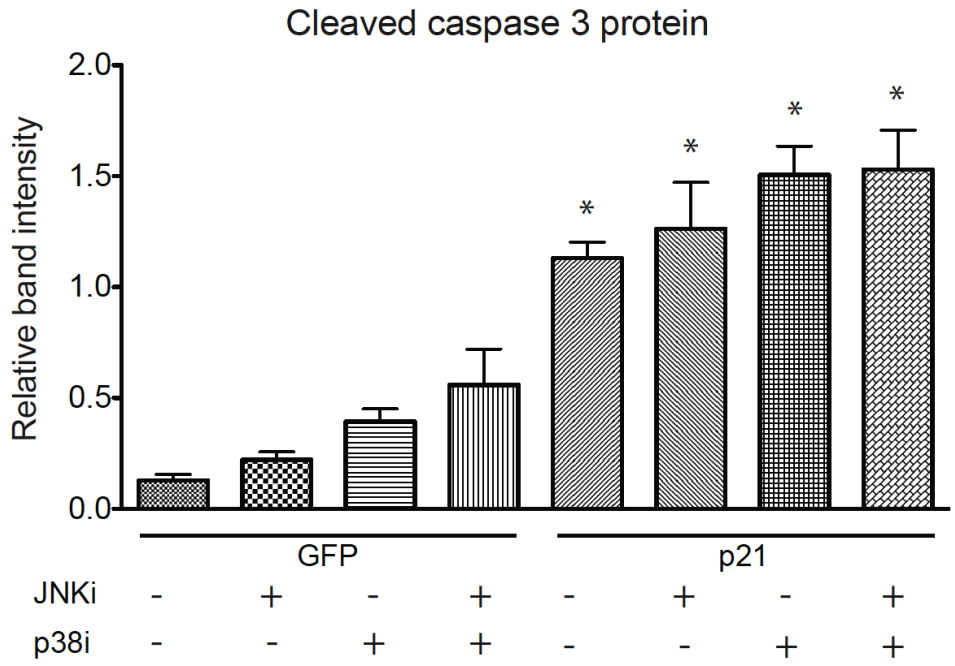


**Figure 20**. **p21-mediated cell death is not dependent on the JNK nor p38 MAPK pathways**. The p38 MAP Kinase inhibitor (p38i) PD169316 was freshly added every 8 h alone or in combination with the c-Jun Kinase inhibitor (JNKi) SP600125 in conditions of either GFP or p21 overexpression. This figure depicts the quantitative analysis of a western blot for cleaved capase 3 normalized to actin. Data are represented as means ± SEM. * indicates significance vs GFP DMSO.

**Similar to glucolipotoxicity, p21 overexpression negatively regulates insulin signaling.**

We next queried whether the induction of p21 during beta cell stress influences classical cell survival mechanisms. Growth factor signaling and insulin signaling both contribute to enhanced proliferation and survival of beta cells (Kasuga 2006, Alejandro 2010, Beith 2008). Therefore, we examined insulin receptor substrate (IRS1), a key mediator of insulin signaling. We observed a reduction of phosphorylated IRS1 with p21 overexpression (**Figure 21A, 21C**). Although high glucose stimulates increased IRS1 activation, we observe a similar decrease in activation of IRS1 with glucolipotoxicity (**Figure 21B, 21D**). Our previous findings also suggest that the downstream Akt activation is also reduced with p21 overexpression (Hernandez et al. 2013). These findings suggest a similar pattern between glucolipotoxicity- and p21-mediated cell death as they both increase stress kinase responses, while decreasing insulin signaling.

**Upregulation of p21 in 832/13 beta cells impairs glucose-stimulated insulin secretion.**

As p21 overexpression impaired insulin signaling, we explored the idea that this effect could be attributable to impaired insulin secretion, which would lead to decreased autocrine/paracrine activation of the insulin receptor and commensurate downstream signaling. As predicted, p21 overexpression completely blunted GSIS (**Figure 22A**). However, it is important to consider that the impaired GSIS could be due to increased cell death evident with p21

overexpression – i.e., a dying beta cell would be unable to appropriately secrete insulin. Therefore, 828/33 cells, which are resistant to p21-mediated cleaved caspase-3/apoptosis, were utilized to rule out death as a contributing factor to impaired GSIS. p21 overexpression again completely impaired GSIS, even without the increase in cell death (**Figure 22B**). Therefore, the upregulation of p21 during stress conditions not only induces intrinsic apoptosis, but it also impairs beta cell function.

**Figure 21.**

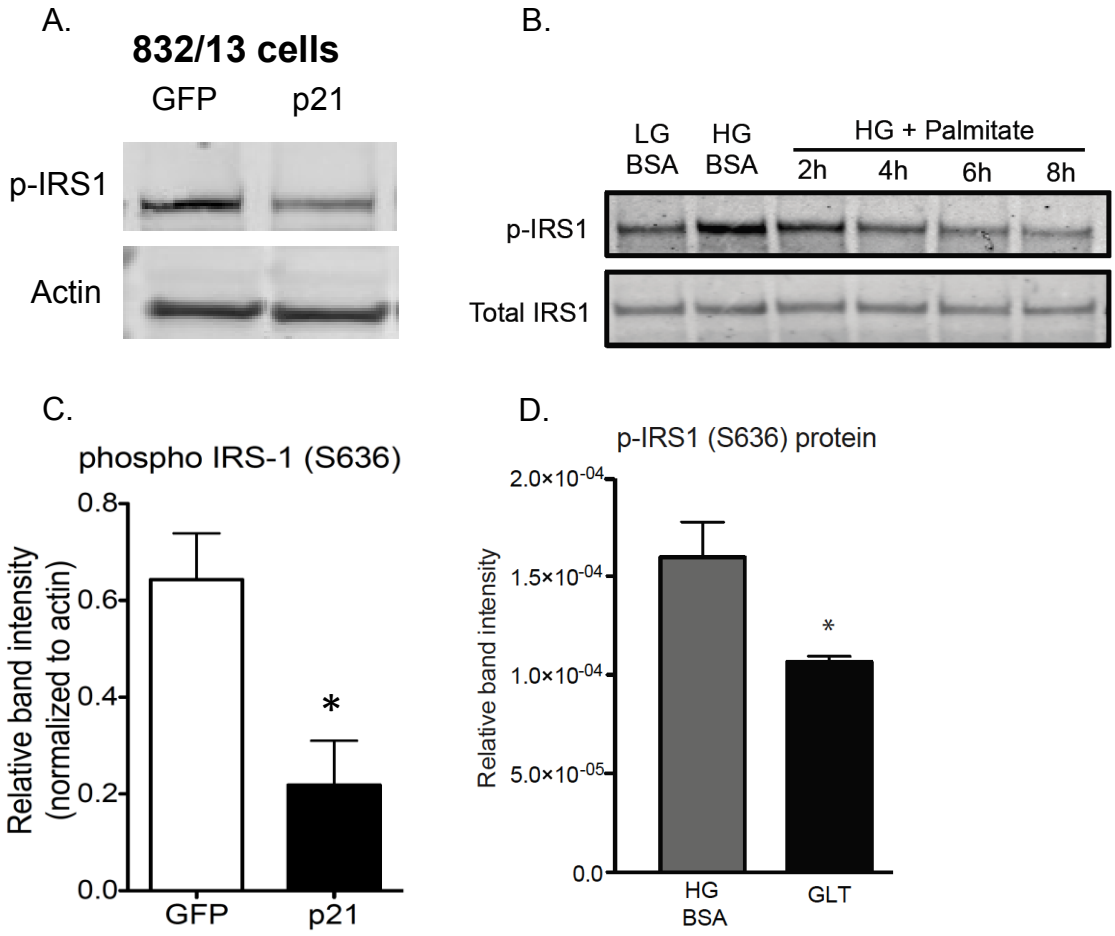


**Figure 21. Insulin signaling is reduced during p21- and glucolipotoxicity-mediated stress.** 832/13 cells were either (A,C) transduced with GFP- or p21-overexpressing adenovirus, or (B,D) treated with glucolipotoxicity for 2, 4, 6, or 8 h. (A-B) Representative western blot of phosphorylated IRS1 (p-IRS1) at S636. Quantitative analysis of p-IRS1 protein during (C) GFP- vs p21-overexpression, or (D) BSA in 25 mM glucose-containing media (HG BSA) vs 400 μM palmitate in 25 mM glucose-containing media (GLT; 4 h). Data are represented as means ± SEM, n=4. * indicates significance vs.GFP or HG BSA, $p<0.05$.

**Figure 22.**

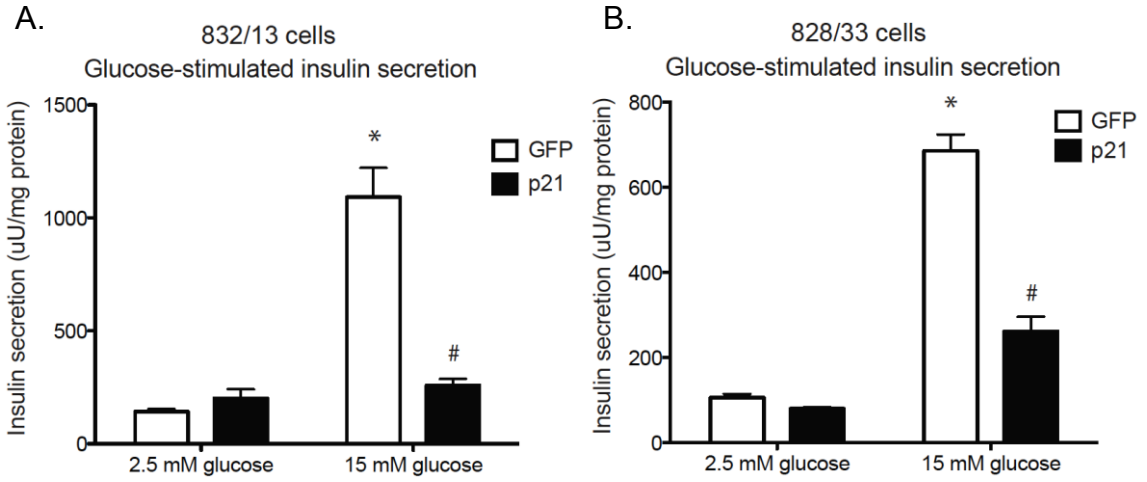


**Figure 22. Upregulation of p21 impairs glucose-stimulated insulin secretion.** (A) 832/13 or (B) 828/33 cells were transduced with GFP- or p21-overexpressing adenovirus for 45 h and underwent a GSIS assay. The incubation buffer from cells was collected after 1 h with exposure to either 2.5 or 15 mM glucose to measure insulin secretion with a radioimmunoactivity assay. Data are represented as means ± SEM, n=3. * indicates significance vs. 2.5 mM glucose, # indicates significance vs GFP at 15 mM glucose, $p<0.05$.

**p21 overexpression reduces FOXO1 phosphorylation and activates expression of its pro-apoptototic target gene, Bim.**

Insulin (and other growth factor) signaling leads to the activation of Akt to trigger downstream cell survival pathways. One immediate downstream action of Akt is to inhibit FOXO1, a transcription factor that is implicated as a key mediator of glucolipotoxicity-induced beta cell stress (Okamoto et al. 2006). FOXO1 transcriptionally activates the expression of the Fas ligand (Fas-L) and the BH3 domain containing Bim, both of which activate apoptosis (Carter and Brunet 2007). Fas-L specifically activates the extrinsic apoptotic pathway, whereas Bim is a mediator of both the intrinsic and extrinsic apoptotic pathways. As we have shown previously that p21 activates the intrinsic and not the extrinsic apoptotic pathway, we thus decided to assess Bim expression. Due to the fact that p21 overexpression reduces insulin signaling and Akt phosphorylation, we predicted that FOXO1 is activated during p21-mediated stress, similar to that which occurs with glucolipotoxic stress. We observed that p21 overexpression in the 832/13 beta cell line significantly decreased FOXO1 phosphorylation (**Figure 23A-B**) and its pro-apoptotic target Bim (**Figure 23C**). As expected, glucolipotoxicity also decreased Fox-O1 phosphorylation **(Figure 24A-B**) and activated gene expression of Bim (**Figure 24C**). These findings again show that glucolipotoxicity and p21 overexpression show similar effects on cell signaling pathways.

**Figure 23.**

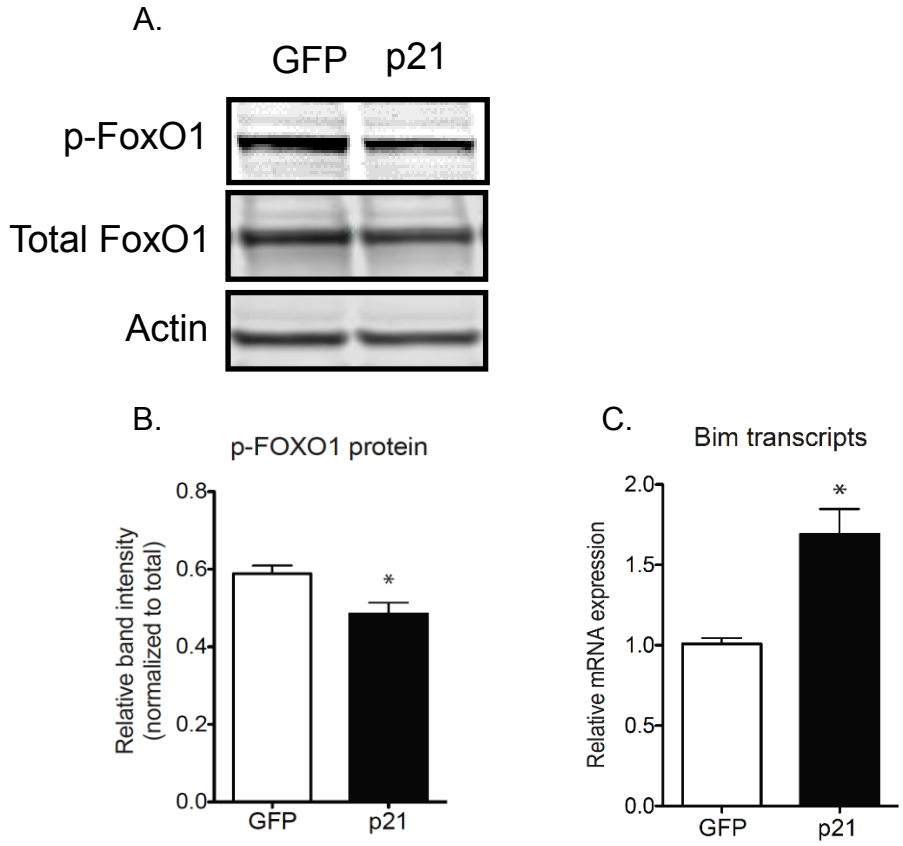


**Figure 23. p21 overexpression reduces phosphorylation of FOXO1 and activates expression of its target gene, Bim**. (A) Western blot analysis of phosphorylated FOXO1 (p-FOXO1) from GFP- or p21-overexpressing 832/13 cells that were transduced for 48 h. (B) Quantification of p-FOXO1 was normalized to total FOXO1. (C) qPCR was used to analyze Bim expression in GFP- or p21-overexpressing 832/13 cells. Data are represented as mean ± SEM, n=3-4. * indicates significance v.s GFP in a student's t-test; $p < 0.05$.

**Figure 24.**

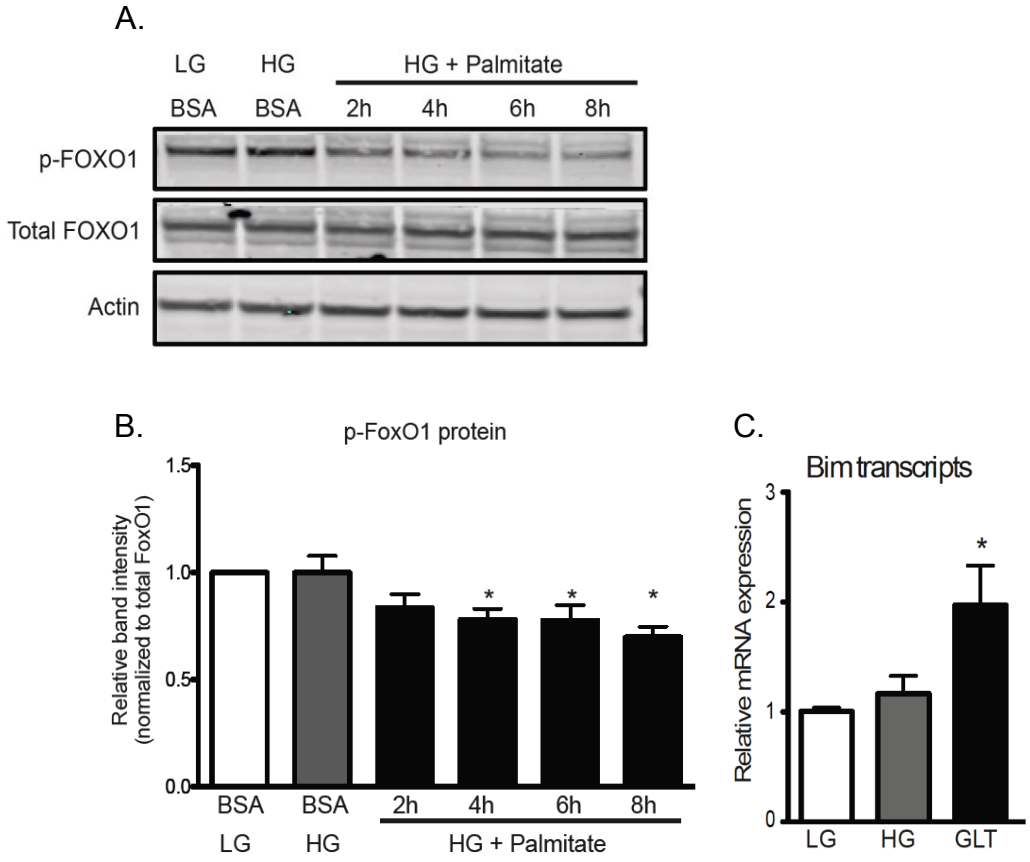


**Figure 24. Glucolipotoxicity reduces phosphorylation of FOXO1 and activates Bim expression.** 832/13 cells were treated with 1% BSA in either 5 mM glucose (LG) or in 20 mM glucose (HG) and compared to cells treated with 400 μM palmitate in 20 mM glucose (GLT). Various treatment times were used as indicated or for 2 h with GLT. (A) Representative western blot images and (B) quantification of FOXO1 (p-FOXO1) normalized to total FOXO1/actin are shown. (C) qPCR was used to measure gene expression of Bim. Data are represented as mean ± SEM, n=3-4. *indicates significance vs. LG BSA in a one-way ANOVA using Tukey's post hoc, $p < 0.05$.

**p21-mediated cell death is independent of Bim expression**.

Recent studies provide evidence that glucotoxicity-induced cell death is reduced in Bim knockout mice (Wali et al. 2014), suggesting that Bim expression is partially responsible for glucolipotoxicity- and ER stress-induced death. Furthermore, Bim expression is upregulated in human islets of patients with T2D. Because upregulation of p21 induced Bim expression, we tested whether Bim was necessary for p21-mediated cell death. Using siRNA-mediated suppression of Bim in conjunction with adenovirally-mediated p21 overexpression, we found that although there was sufficient knockdown of Bim expression (**Figure 25A, 25C**), p21 overexpression still induced the same amount of cell death as measured by cleaved caspase 3 protein (**Figure 25B, 25D**). These data indicate that Bim is not required for p21-mediated cell death.

**p21 is necessary for ER-stress induced cell death**.

Both glucolipotoxicity- and thapsigargin-induced ER stress upregulate p21. Thus we determined the extent to which p21 is necessary for ER-stress induced cell death. Using p21 knockout mice (p21 KO), we treated their islets *ex vivo* with thapsigargin and observed no difference in ER stress response as indicated by similar CHOP expression (**Figure 26A**). However, when examining apoptosis via a DNA fragmentation assay, thapsigargin treated wild-type islets resulted in a 2.5-fold increase in apoptosis, whereas in p21 KO islets, we observed a blunted thapsigargin-induced cell death response (**Figure 26B**). These data suggest that p21 required, in part, for thapsigargin-induced cell death in beta cells.

**Figure 25.**

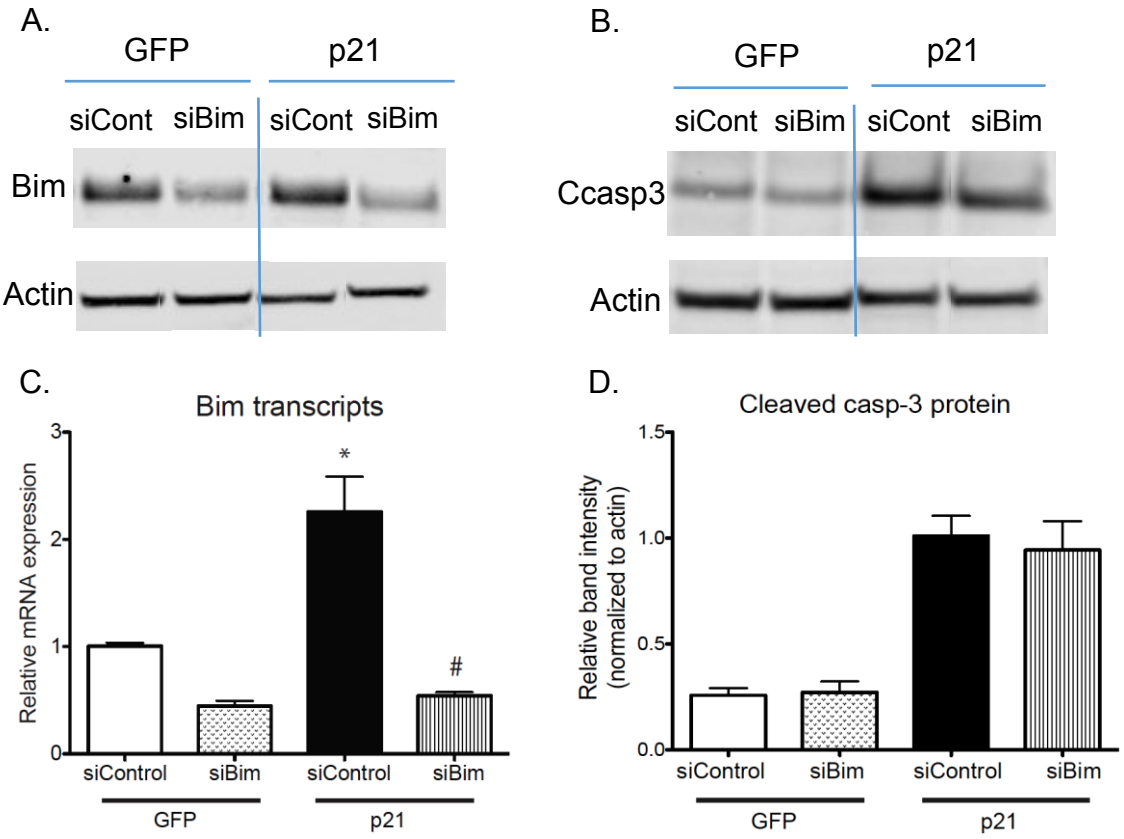


**Figure 25. Bim suppression does not reduce p21-mediated cell death.**

832/13 cells underwent siRNA-mediated suppression of Bim in the presence of either a GFP- or p21-overexpressing adenovirus. (A) Representative western blot image of Bim protein and (C) qPCR of Bim gene expression are shown. (B) Representative western blot image of cleaved caspase 3 (Ccasp3) and (D) quantitative analysis of Ccasp3 protein are also shown. Data are represented as means ± SEM, n=4. * indicates significance vs siControl + GFP-overexpressing adenovirus, # indicates significance vs siControl + p21-overexpressing adenovirus, $p<0.05$.

**Figure 26.**

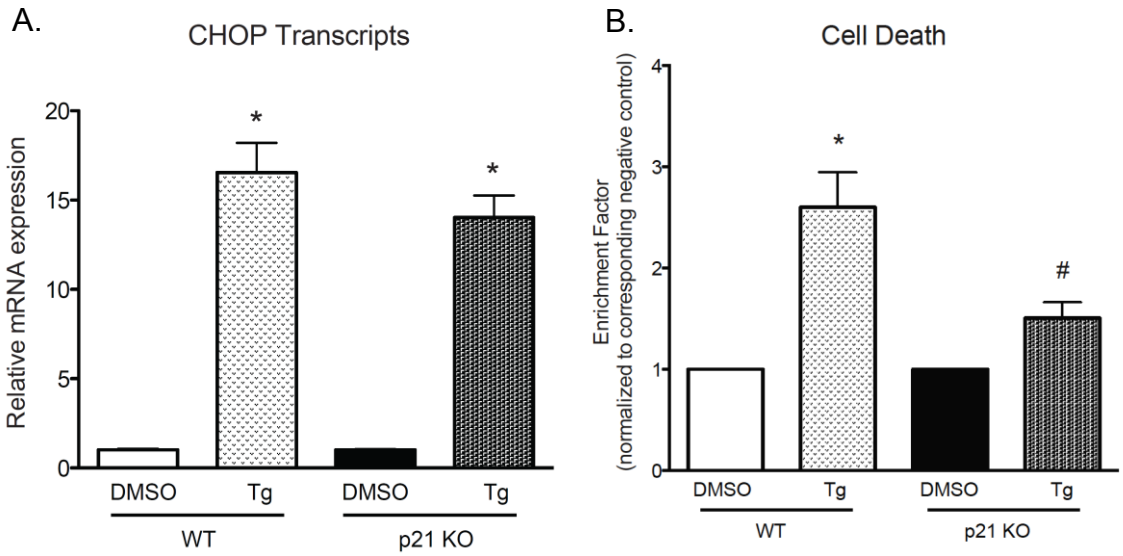


**Figure 26. p21 knockout mice exhibit lower ER-stress induced death.** Isolated mouse islets from homozygous p21 wild-type (WT) or knockout (KO) mice were treated with DMSO or 10 μM thapsigargin (Tg) for 48 h. qPCR was used to measure (A) CHOP gene expression. (B) Additionally, an ELISA Cell Death assay was used to quantify DNA fragmentation in WT or p21 KO mice. Data are represented as mean ± SEM, n=4 for each genotype. * indicates significance vs DMSO within genotype, # indicates significance vs WT treated with Tg, $p<0.05$.

## IV.D. Discussion

Most individuals with T2D and even late pre-diabetes have elevated circulating glucose levels and impaired insulin-mediated inhibition of lipolysis, creating a glucolipotoxic milieu within the individual. Glucolipotoxicity in the pancreatic beta cell can lead to the demise of beta cell function and beta cell mass. In fact, death of beta cells is triggered by glucolipotoxicity as the increased lipids incorporate into the ER membrane and in combination with high glucose, activate the UPR, the production of ROS, and the induction of inflammation. In addition, glucolipotoxicity impairs beta cell function and insulin secretion. In our findings, we confirm that glucolipotoxicity indeed activates ER stress, leads to increased phosphorylation of stress responsive kinases, and impairs insulin and cell survival signaling. Interestingly, we also discovered that glucolipotoxicity induces expression of the cell cycle inhibitor, p21.

Glucolipotoxicity is one of many insults that the beta cell faces during course of diabetes, and it is important to determine similarities between the mechanisms leading to cell death in order to find new therapeutic approaches to protect beta cells from these insults. Our previous findings demonstrated that other beta cell stressors, such as glucocorticoid receptor activation by dexamethasone and ER stress by the SERCA inhibitor thapsigargin, both also upregulated p21 expression. Additionally, both chronic high glucose and intermittent high glucose also induce p21 expression (Zhang, Li, et al. 2014). In further support, p21 mRNA was significantly increased in a microarray analysis of islets from mice fed a high fat diet compared to mice on chow diet (Sims et al.

2013). Therefore, under a cadre of diverse stress conditions, p21 is upregulated in the beta cell.

Similar to glucolipotoxicity, p21 overexpression activates the stress responsive JNK at the earliest time point examinined (i.e., 2 h). As previously described, phosphorylation of JNK is an early event that diminishes over time and occurs within 1 h of high concentrations of the fatty acid palmitate (Martinez et al. 2008). Therefore, JNK is an early participant of glucolipotoxicity-induced signaling that leads to beta cell death. Interestingly, inhibitors of JNK reduced glucolipoxity-induced caspase 3 cleavage (Martinez et al. 2008). However, in the work presented here, inhibition of JNK did not blunt p21-mediated cell death. This suggests that p21 overexpression activates JNK, an upstream event to death that could influence other cellular signals. The insulin signaling pathway is one that is reduced by the activation of JNK, and is reversed when JNK is inhibited (Nguyen et al. 2005). Thus, we decided to further investigate the influence of p21-mediated JNK activation on the insulin signaling pathway.

Our findings show that p21 overexpression also reduced insulin signaling, and specifically reduced p-IRS1 and p-Akt. Interestingly, JNK inhibits IRS-1 by associating with it and phosphorylating the Serine 307 site to promote insulin resistance (Aguirre et al. 2000). Thus, further studies could determine whether this event is occurring during conditions of p21 upregulation. Given that the insulin signaling pathway is diminished during p21 overexpression, we postulate that this is one primary mechanism by which p21 increases beta cell death.

We next investigated whether the impaired insulin signaling could be attributed to changes in the upstream GSIS under these conditions. Observations indicate that aside from activation of stress kinases and impaired insulin signaling, upregulation of p21 in beta cells resulted in impaired GSIS. However, the exact mechanism of p21's action to impair GSIS remains to be determined.

Akt activation enhances cell survival signaling by inhibiting FOXO1 nuclear import where it can transactivate expression of pro-apoptotic proteins such as Bim and FASL. Although FOXO1 can confer stress resistance during JNK activation (Matsumoto and Accili 2005), chronic FOXO1 activation is part of the mechanism for glucolipotoxicity of beta cells (Martinez et al. 2008, Banks et al. 2011, Accili and Arden 2004). Here, we discovered that like glucolipotoxicity, p21 overexpression resulted in a decrease in the Akt-phosphorylated site on FOXO1. Therefore, FOXO1 is capable of nuclear import and activation of transcriptional activity. However, FOXO1 transcriptional activity is yet to be measured during p21 overexpression.

In our findings, we also discovered that the BH3 domain-containing protein Bim, a FOXO1 target gene, is upregulated during glucolipotoxicity and p21 overexpression. Interestingly, islets of Bim knockout mice are partially protected from glucotoxicity and along with another proapoptotic BH3-only protein Puma, is postulated as one of the downstream effectors glucotoxicity-induced ER stress and mitochondrial oxidative stress (Wali et al. 2014). Although our experiments using Bim knockdown cells did not show protection from p21-mediated cell

death, induction of Bim could be one of multiple effectors that p21 acts on that contributes to the demise of beta cells.

An important discovery from this work is that knockout of p21 partially protects from cell death in response to increased ER stress. In other cell types, p21 has been linked to an anti-apoptotic role; however, in beta cells, our findings lead us to the proposed model where beta cell stressors upregulate p21 expression to act on multiple effectors that lead to the demise of function and survival (**Figure 27**). Specifically, the upregulation of p21 in beta cells is partially responsible for the activation of JNK, FOXO1, and Bim expression, whereas at the same time contributes to the inhibition of insulin signaling and IRS-1, and including the downstream cell survival signaling through Akt. Overall, we demonstrated that aside from its classical role of halting proliferation, p21 impairs beta cell function, inhibits beta cell survival, and activates pro-apoptotic signaling pathways.

**Figure 27.**

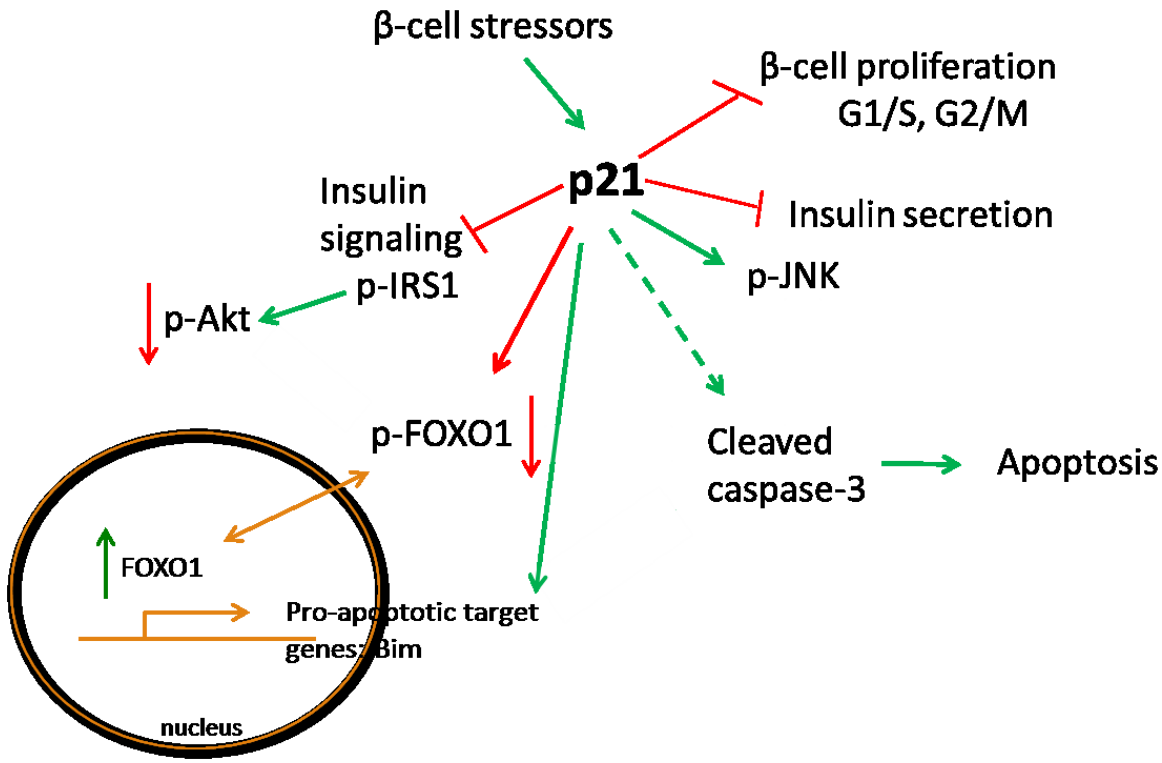


**Figure 27. Proposed model of p21-mediated regulation of beta cell function and survival.** p21 is upregulated under beta cell stress conditions and is partially responsible for halting beta cell proliferation, blunting insulin secretion, inhibiting insulin signaling, and activating stress responses that lead to apoptosis. Specifically, p21 reduces a key regulator of cell survival, Akt, which then allows for expression of FOXO-1's pro-apoptotic gene target, Bim. In addition, p21 induces phosphorylation of the stress activated kinase, JNK. Both Bim and JNK induce apoptosis mediated through cleaved caspase 3. Negative regulation is depicted in red, and positive regulation is depicted in green.

# Chapter V. Conclusions, Perspectives, and Future Studies

## V.A. Conclusions and Perspectives

The main conclusions from this dissertation project are that multiple stressors in the beta cell upregulate p21 expression and that p21 contributes to ER-stress induced intrinsic apoptosis by regulating effectors of the cell survival and cell death pathways. Although the specific mechanism by which p21 regulates these effectors still remains unclear, the findings from my work suggest that p21's actions occur downstream of ER stress but upstream of the activation of the stress kinases and/or inhibition of insulin signaling and impaired beta cell function.

Stressors such as cytokines, glucocorticoids, and gluco(lipo)toxicity, contribute to beta cell destruction through various and sometimes overlapping mechanisms. My thesis demonstrates that dexamethasone, thapsigargin, and glucolipotoxicity, which have distinct modes of action to induce beta cell stress, all induce p21 expression. Stress responses activated in dying beta cells include inflammation, ER stress, and ROS. Although my primary work investigated ER stress, others found that reactive oxygen species also induce p21 expression in beta cells (Kaneto, Kajimoto, Fujitani, et al. 1999). Another noteworthy finding is that 832/13 cells incubated with proinflammatory cytokines did not induce p21 expression. This result signifies that not all mechanisms of stress-induced beta cell death, such as inflammation, are regulated by p21. However, with my findings and those of others (Kaneto, Kajimoto, Fujitani, et al. 1999, Taneera et

al. 2013, Hughes and Huang 2011, Ranta et al. 2011, Tarry-Adkins et al. 2009, Yamada et al. 2006, Zhang et al. 1999, Zhang, Li, et al. 2014), p21 is a common factor among numerous stressors that warrants further investigation.

The major finding for Chapter III is that p21 activates the intrinsic apoptotic pathway rather than the extrinsic pathway. During the first screen of mitochondrial proteins of the Bcl-2 family, we did not identify any changes in expression of these proteins with p21 overexpression. However, we later determined in Chapter IV that Bim, which was not included in the original screen, is a mitochondrial protein in the Bcl-2 family that is upregulated with p21 overexpression. Therefore, my findings suggest that p21 is activating the intrinsic pathway via upregulation of Bim expression. However, we later determined that knockdown of Bim alone was unable to block p21-mediated cell death.

Bim is a BH3 domain-only pro-apoptotic protein, like Bax and Bak. Although Bim does not have the capability for pore formation on the mitochondrial membrane, unlike Bax and Bak, it is able to anchor onto the mitochondrial membrane to antagonize Bcl-2 (Gross, McDonnell, and Korsmeyer 1999). Both glucolipotoxicity and p21 overexpression induce Bim expression. Interestingly, we found that knockdown of Bim was not sufficient to inhibit p21-mediated caspase 3 cleavage. However, overexpression of Bcl-2 was sufficient to block p21-mediated cell death. Furthermore, knockdown of Bax and Bak, the pore forming mitochondrial proteins, also reduce p21-mediated death. This suggests that by knocking down Bim, p21 is still able to stimulate pore formation independent of Bim at the membrane and that p21 mediated death is dependent

on the pore forming unit. The studies I performed to investigate expression of these proteins on the mitochondrial membrane were inconclusive due to technical difficulties, however, I suspect that although transcription of Bcl-2, Bax, and Bak did not change, their translocation to the mitochondrial membrane are dynamically regulated during conditions of p21 overexpression.

The studies from Chapter III also hinted towards findings of decreased cell survival signaling, as indicated by the decrease in phosphorylated Akt under conditions of p21 overexpression. We dove a little deeper into the insulin signaling pathway in Chapter IV and found that p21 overexpression also decreased phosphorylated IRS-1, a substrate upstream of Akt and downstream of the insulin receptor. This led us to examine GSIS as a possible explanation for the reduced insulin and pro-survival signaling. Overexpression of p21 indeed impaired GSIS in both the 832/13 and 828/33 beta cells. The 828/33 cells are resistant to p21-mediated cell death and thus allowed us to examine p21-mediated impairments of GSIS independent of death-associated impairments of GSIS. Interestingly, previous work by others determined that p21 inhibits insulin gene expression and GSIS (Kaneto, Kajimoto, Fujitani, et al. 1999, Dayeh et al. 2014). It would be ideal to verify whether insulin content or gene expression is regulated by p21.

Due to the fact that glucolipotoxicity is classically known to activate FOXO1, and the fact that it is an immediate downstream target of Akt, I next examined FOXO1. Chronically activated FOXO1 is responsible for the production of pro-apoptotic proteins. Findings in Chapter IV verify that FOXO1 is activated

with both glucolipotoxicity and p21 overexpression, as measured by decreased FOXO1 phosphorylation. Others have indicated that FOXO1 can also be activated by acetylation (Banks et al. 2011, Qiang, Banks, and Accili 2010). It would be interesting to determine whether p21 regulates the acetylation of FOXO1 as well. Previous studies by Martinez et al. show that the activation of FOXO1 with glucolipotoxicity is associated with translocation of FOXO1 into the nucleus (Martinez et al. 2008). In order to determine whether the influence of p21 on FOXO1 activation is critical for the pro-apoptotic effect of p21, it would be ideal to overexpress the dominant negative form of FOXO1 in the presence of p21 overexpression, or alternatively utilize beta cell speficic FOXO1 knockout mice. If p21-mediated cell death is reduced under such conditions, this would suggest that FOXO1 activation is essential to the pro-apoptotic mechanism of p21.

In our studies that examined whether p21-mediated death was dependent on JNK activation, we used SP600125, a selective and reversible JNK1, -2, and -3 inhibitor that works by competing with ATP to inhibit the phosphorylation of c-Jun (Bennett et al. 2001). One caveat to these studies is that although we saw decreased phosphorylation of the JNK target protein c-Jun, we did not see a significant decrease in p-JNK, indicating that there was still some active JNK available for signaling. An alternative approach to solve this issue is to use a dominant negative JNK (DN-JNK1) in 832/13 cells to inhibit JNK functionality. Our recent findings show that DN-JNK1 inhibited thapsigargin-induced cell death. The next step would be to determine whether active JNK is necessary for p21-

mediated cell death. These future experiments would give either verification of our previous results or lead to further insight on the mechanistic details of p21 action after stressful insults within the beta cell.

Importantly, we have shown both similarities and differences in the cellular events that occur with thapsigargin-, glucolipotoxicity- and p21 overexpression-induced beta cell stress. Specifically, thapsigargin and p21 overexpression both halt proliferation, lead to phosphorylation of eIF2α, and induce cleaved caspase 3 in 832/13 cells. Similiarly, in 828/33 bcl-2 overexpressing cells, thapsigargin and p21 overexpression lead to elevated phosphorylation of eIF2α and a blockage of cleaved caspase 3. Additionally, thapsigargin treatment, glucolipotoxicity, and p21 overexpression lead to phosphorylation of JNK and upregulation of CHOP expression. On the contrary to thapsigargin- and glucolipotoxicity-induced beta cell stress, p21 overexpression does not increase spliced Xbp-1. Lastly, inhibiting JNK activation reduces thapsigargin-induced cleaved caspase 3, whereas JNK inhibitors did not inhibit p21 overexpression mediated cleaved caspase 3.

The overall conclusions of this dissertation are that p21 regulates multiple responses in the face of stress, including its classical role of halting cell cycle progression, activating the PERK arm of the UPR, activating stress kinases and FOXO1, impairing GSIS, and reducing insulin signaling and cell survival responses. However, future experiments need to determine the exact mechanism by which p21 intereferes with these signaling events. It would be ideal to do a rapid knockdown study of each signaling intermediate and determine whether deleterious effects of p21 are still observed. Signaling

intermediates would include those involved in insulin signaling, the UPR, MAPK pathways, Akt/mTOR pathways, FOXO-1 pathways, and the mitochondrial intrinsic apoptotic pathway. Furthermore, studies on how p21 directly acts on these intermediates is needed. For example, does p21 bind and inhibit these intermediates? What is the mode of action by which p21 regulates expression or phosphorylation of these proteins?

Given that deleterious effects on beta cells were observed in my project, p21 may be a new target to further examine to prevent diabetes. It would be interesting, however, to examine whether p21 plays a similar role in these processes in islets of donors with other forms of diabetes, such as type 1 diabetes or gestational diabetes. ER stress is also a postulated mechanism that is thought to be involved in the progression of type 1 diabetes (Marhfour et al. 2012, Hopfgarten et al. 2014), and thus p21 may also be induced in these islets. If so, this further validates the necessity for making a p21 inhibitor that specifically targets the beta cell. Currently, there is no selective inhibitor of p21, and even more challenging is creating a beta cell-specific targeting drug. However, there are current tools, such as bioengineered linking of two hormones, that are now exploited in hopes to create such drugs and improve targeted delivery (Finan et al. 2012). As alternative, one can envision targeting the deleterious targets of p21 itself (e.g., blocking or restoring the mechanism of how p21 inhibits insulin secretion and signaling).

## V.B. Future Studies

Future studies that need further investigation that are directly related to this project are the effects that p21 had on p-eIF2α, due to the fact that the UPR is known to mediate cell cycle arrest, and the activation of eIF2α leads to attenuation of translation. However, in this case we observed that the cell cycle inhibitor is mediating eIF2α activation. This brings a couple questions to mind: is it possible that p21 overexpression will lead to the inhibition of global translation as well? Or alternatively, is phosphorylation of eIF2α after p21 overexpression a potential negative feedback mechanism to protect the cell from producing too much p21? The expression of proteins involved in the cell cycle machinery during stress must be tightly regulated in order to prevent mutations and cancerous growths. Therefore, p21's effect on eIF2α could be a mechanism tying in the levels of cell cycle regulators to the translation machinery. In other words, signals that halt the cell cycle could be activating the UPR as a signal that there is a problem with the production of proteins.

Another question to consider is whether p21 is negatively affecting insulin protein translation or folding and whether this is the cause for the increase in phosphorylated eIF2α under conditions of p21 overexpression. If p21's main action is to inhibit homeostatic levels of insulin, it could be the main mechanism by which beta cell death is occurring. Insulin directly acts on beta cells for cell survival. Therefore, with a decrease in insulin production and GSIS, is p21 overexpression diminishing the necessary beta cell survival signals from insulin? Future studies should undergo insulin restoration during conditions of p21

overexpression to examine whether p21-mediated cell death is diminished and insulin signaling restored.

Lastly, experiments utilizing p21 knockout mice in conditions of high fat diet should be used to recapitulate the same conclusions that were determined from *in vitro* glucolipotoxicity experiments. For example, do p21 knockout mice fed a high fat diet have improved outcomes on insulin signaling, or death compared to WT mice on high fat diet? If so, does this lead to an overall improvement in functional beta cell mass? Determining whether knocking out p21 is beneficial in terms of functional beta cell mass will help us determine whether future clinical studies should be performed using p21 inhibitors.

Aside from these prospective studies directly related to my project, findings from others have also provoked interest in novel aspects of functional beta cell mass. For example, DNA methylation and the epigenetics in islets of donors with T2D are differentially expressed, and is even evident with the p21 loci (Dayeh et al. 2014). Further extension of understanding the epigenetic regulation in different types of diabetes could give further insight as to how functional beta cell mass is regulated in each disease state, and which signaling pathways are causing these changes. Given that epigenetic changes occur even before birth, they may be the cause for predisposition to a certain type of diabetes and perhaps be one of the emerging fields of interest that will set the stage for new and upcoming theories on functional beta cell mass.

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improved recovery from streptozotocin-induced diabetes." *PLoS One* 4 (12):e8344. doi: 10.1371/journal.pone.0008344.
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Zhang, Y., N. Fujita, and T. Tsuruo. 1999. "p21Waf1/Cip1 acts in synergy with bcl-2 to confer multidrug resistance in a camptothecin-selected human lung-cancer cell line." *Int J Cancer* 83 (6):790-7.
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Zhou, B. P., Y. Liao, W. Xia, B. Spohn, M. H. Lee, and M. C. Hung. 2001. "Cytoplasmic localization of p21Cip1/WAF1 by Akt-induced phosphorylation in HER-2/neu-overexpressing cells." *Nature cell biology* 3 (3):245-52. doi: 10.1038/35060032.
Zong, W. X., and C. B. Thompson. 2006. "Necrotic death as a cell fate." *Genes Dev* 20 (1):1-15. doi: 10.1101/gad.1376506.

**Curriculum Vitae**

**Angelina M. Hernández-Carretero**

## EDUCATION

**Indiana University, Indianapolis, Indiana, (IUPUI Campus)**
August 2009-October 2014
**Ph.D.,** Cellular and Integrative Physiology, Diabetes and Obesity minor
Thesis: Novel Roles of p21 in Apoptosis During Beta-cell Stress in Diabetes

**California State University, Los Angeles, California**
August 2007-September 2009
**M.S.,** Biological Sciences

**University of California, Irvine, California**
September 2003-June 2007
**B.S.,** Biological Sciences

## FELLOWSHIPS

University of California, San Diego
IRACDA Postdoctoral Fellowship
October 2014-October 2017

American Physiological Society
William Porter Physiology Development Fellowship
August 2012-August 2014

Indiana University School of Medicine
Edwin T. Harper Scholar Fellowship
August 2011-February 2012

California State University, Los Angeles
LSAMP Bridge to the Doctorate Fellowship, Cohort V
August 2007-August 2009

## PUBLICATIONS

### MANUSCRIPTS

**Hernandez AM**, Colvin ES, Olivos N, Fueger PT, *Glucolipotoxicity-induced p21 suppresses pro-survival signaling in pancreatic beta cells*. *(In Preparation)*

**Hernandez AM**, Colvin ES, Chen Y-C, Geiss SL, Eller LE, Fueger PT, *Upregulation of p21 activates the intrinsic apoptotic pathway in beta cells*. *AM J Physiol Endocrinol Metab, 2013. 304 (12): E1281-90.*

Colvin ES, Ma H-Y, Chen Y-C, **Hernandez A**, Fueger PT, *Glucocorticoid-induced suppression of beta cell proliferation is mediated by Mig6*. *Endocrinology, 2013. 154 (3): 1039-46.*

Fueger PT, **Hernandez AM**, Chen Y-C, Colvin ES. *Assessing replication and beta cell function in adenovirally-transduced isolated rat islets*. *J Vis Exp, 2012. 25 (64): 4080.*

Roozendaal B, **Hernandez A**, Cabrera SM, Hagewoud R, Malvaez M, Stefanko DP, Haettig J, Wood MA, *Membrane-associated glucocorticoid activity is necessary for modulation of long-term memory via chromatin modification. J Neurosci*, 2010. 30 (14): 5037-46.

## ABSTRACTS

**Hernandez AM**, Colvin ES, Fueger PT, Glucolipotoxicity-induced p21 suppresses pro-survival signaling in pancreatic beta cells. 2014 Experimental Biology, FASEB J. 28: 1108.9.

**Hernandez AM**, Colvin ES, Fueger PT, Upregulation of p21 activates the intrinsic apoptotic pathway in pancreatic beta cells. 2013, Experimental Biology, FASEB J, 27:1154.22.

**Hernandez AM**, Colvin ES, Fueger PT, Cyclin-dependent kinase inhibitor 1 initiates apoptosis in beta cells and rat islets. 2012, Society for the Advancement of Chicanos and Native Americans in Science National Conference.

**Hernandez AM**, Jeffries K, Colvin ES, Fueger PT, Trefoil Factor 3 is not required to establish functional beta cell mass in vivo. 71st Scientific Session American Diabetes Association.

**Hernandez A**, Cho J, Contreras J, Russo-Neustadt AA, Exercise prevents stress associated effects on hippocampal-dependent spatial learning in the Barnes Maze. Society for Neuroscience 38th Annual Meeting.

## PROFESSIONAL CONFERENCES ATTENDED

- Experimental Biology Meeting
  April 2014
  San Diego, CA
- Institute on Teaching and Mentoring Annual Conference

October 2013
Arlington, VA

- National Organization for the Professional Advancement of Black Chemists and Chemical Engineers 40th National Meeting
  October 2013
  Indianapolis, IN
- Experimental Biology Meeting
  April 2013
  Boston, MA
- 3rd Annual Indiana Physiological Society Meeting
  February 2013
  Indianapolis, IN
- Society for the Advancement of Chicanos and Native Americans in Science National Conference
  October 2012
  Seattle, WA
- Midwest Islet Biology Club 5th Annual Meeting
  May 2012
  Pittsburgh, PA
- National Organization for the Professional Advancement of Black Chemists and Chemical Engineers 40th National Meeting
  November 2011
  Indianapolis, IN
- 1st Annual Indiana Physiology Society Meeting
  February 2011
  Indianapolis, IN
- Preparing Future Faculty Symposium
  November 2011
  Indianapolis, IN
- 71st Scientific Sessions of the American Diabetes Association
  June 2011
  Orlando, FL
- Midwest Islet Biology Club 4th Annual Meeting
  May 2011
  Madison, WI
- Institute on Teaching and Mentoring Annual Conference
  October 2010
  Tampa, FL

## PROFESSIONAL AFFILIATIONS

- The Endocrine Society, Student Member
  2014-2015
- American Physiological Society, Student Member
  2011-2014

- National Organization for the Professional Advancement of Black Chemists and Chemical Engineers, Student Member
  2013-2014
- Underrepresented Professional and Graduate Student Organization, Student Member
  2009-2014
- Indiana Physiology Society, Student Member
  2010-2014
- American Association for the Advancement of Science, Student Member
  2011-2013
- Society for Advancement of Chicanos and Native Americans in Science, Life Member
  2010-2014

**PRESENTATIONS**

- Poster Presentation
  April 2014
  Experimental Biology
- Oral Presentation
  April 2014
  Experimental Biology-APS Endocrinology & Metabolism Section Featured Topic
- Research Poster Presentation
  February 2014
  Indiana Biomedical Gateway Program Recruitment Poster Session
- Oral Presentation
  December 2013
  Metabolism and Islet Biology Seminar Series
- Oral Presentation
  October 2013
  Indiana University Center for Diabetes Research Seminar Series
- Poster Presentation
  April 2013
  Experimental Biology
- Oral Presentation
  February 2013
  3rd Annual Indiana Physiological Society Meeting
- Thesis Proposal Oral Presentation
  February 2013
  Department of Cellular & Integrative Physiology Seminar Series
- Research Poster Presentation
  February 2013
  Indiana Biomedical Gateway Program Recruitment Poster Session
- Oral Presentation
  October 2012

SACNAS National Conference

- Poster Presentation
  May 2012
  Midwest Islet Biology Club 5th Annual Meeting
- Poster Presentation
  November 2011
  NOBCChE Midwest Regional Health Science and BioTechnology Conference
- Research Poster Presentation
  June 2011
  71st Scientific Sessions of the American Diabetes Association

## AWARDS

- University of California, San Diego
  2014
  Institutional Research and Academic Career Development Award
- Indiana University School of Medicine-Physiology Department
  2014
  Dr. Stier Award
- American Physiological Society-Endocrinology and Metabolism Section
  2014
  Research Recognition Award
- American Physiological Society
  2014
  Minority Travel Award
- Indiana Physiological Society's 3rd Annual Meeting
  2013
  Outstanding Graduate Student Abstract
- American Physiological Society-Endocrinology and Metabolism Section
  2013
  Virendra B. Mahesh Award of Excellence in Endocrinology
- Federation of American Societies for Experimental Biology
  2013
  MARC Travel Award
- IUPUI Graduate and Professional Student Government
  2013
  Educational Enhancement Grant
- American Physiological Society
  2013
  Minority Travel Award
- Federation of American Societies for Experimental Biology
  2012
  MARC Travel Award

- IUPUI Graduate and Professional Student Government
  2011
  Educational Enhancement Grant
- IUPUI Graduate and Professional Student Government
  2011
  C3 Outstanding Service to Career, Campus and Community Award

## TRAINING EXPERIENCES

Indiana University School of Medicine
August 2009-September 2014
Department of Cellular and Integrative Physiology
Mentor: Patrick T. Fueger, Ph.D.

California State University, Los Angeles
August 2007-September 2009
Department of Biological Sciences
Mentor: Amelia Russo-Neustadt, Ph.D.

University of California, Irvine
June 2005-June 2007
Center for the Neurobiology of Learning and Memory
Mentor: James McGaugh, Ph.D. and Benno Roozendaal, Ph.D.

## TEACHING EXPERIENCE

Indiana University School of Medicine
F503-Human Physiology
November 2013
Guest Lecturer: “GI motility, chewing, swallowing” and “The Stomach”

Martin University
Human Anatomy and Physiology
February 2013
Guest Lecturer: “The Endocrine System”

Indiana University Brain Link/MASH (K-12) Science Summer Program
Spanish Enrichment Program
July 2011
Enrichment Program Facilitator: “Spanish”

California State University, Los Angeles
Animal Biology
January 2009-June 2009
Lab instructor

## PROFESSIONAL AND UNIVERSITY SERVICE

- Board of Directors, Graduate Student Member, Society for the Advancement of Chicanos and Native Americans in Science
  2014-2016
- Trainee Advisory Committee, Teaching Section Representative, American Physiological Society
  2014-2017
- Executive Board Member, Treasurer, Underrepresented Professional and Graduate Student Organization
  2012-2014
- Preparing Future Faculty Mentor Program
  2011-2014
- Indiana University School of Medicine Student Mentor
  2010-2014
- Graduate and Professional Student Government
  2013-2014
  IU School of Medicine Representative
- Indiana Physiological Society
  2014
  Abstract Reviewer
- Indiana University School of Medicine Graduate Committee
  2013-2014
  Student Representative
- Indiana University School of Medicine Student Ambassador
  2010-2014
  Student Coordinator for Campus Visits
- Indiana University School of Medicine Getting You into IUPUI
  2010-2014
  Student Ambassador
- IUPUI Preparing Future Faculty and Professionals
  2012
  Session Moderator, “Of Color in the Academy”
- Wells Center Molecular Medicine in Action
  2010-2014
  Team Member, “Dissecting Diabetes Module”
- IU Biomedical Gateway Ph.D. Program Diversity Committee
  2009-2013
  Student Representative